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THE INTESTINAL FLORA OF THE GUINEA PIG¹

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In reviewing the earlier work on intestinal flora one notes that the animals which served as subjects were, with few exceptions, omnivorous. The fact that the guinea pig is herbivorous appeared to the present writers to be ample reason for engaging in the present study. Furthermore, the guinea pig is one of the most commonly used experimental animals, and it would seem that a knowledge of its intestinal flora should be of considerable value to investigators. Such an assumption is already being borne out by requests for information about the intestinal flora of so-called normal guinea pigs.

Baumatz (1925) observed only a few bacterial species in the guinea pig, members of the subtilis group and coliform organisms being the most frequent. Baker and Martin (1937-1938) investigated the rôle of bacteria in the breakdown of ingested cellulose. The search was limited to a study of stained smears. Reference was made by them to "*B. caviae*," a large gram-positive sporulating aerobe, first described by Schussing in 1921.

In recent years some attention has been given to a study of bacteria occurring in the different levels or segments of the digestive tract of animals, with results that were more or less conflicting. Porter and Rettger (1940) found the intestinal flora of the white rat relatively simple. The upper segments harbored acidophilus-like forms as the dominant organism. The numbers of bacteria were decidedly larger in the lower sections, *Escherichia coli* becoming more and more common. Contrary to much prevailing opinion, the stomach and upper reaches of the intestine of the albino rat contained appreciable numbers of viable bacteria, under the dietary regimens to which the animals were subjected. Alteration of the flora could be brought about by certain changes of diet.

EXPERIMENTAL

Stock guinea pigs were obtained from three different animal breeders within a few miles of New Haven. As soon as the animals were received at the laboratory they were placed on a stock diet of oats, lettuce and water. A cage was prepared for each animal, which facilitated the collection of fecal samples. Clean but not sterile paper lined the dropping pans under the cages. Feed cups were suspended by wires from the tops of the cages, thus preventing the animals from contaminating the feed with their excreta.

The choice of the proper media for the cultivation of the intestinal bacteria was based upon the experiences of Porter and Rettger (1940) and upon the results of a comparative study of several media not employed by them.

¹ This paper covers in part the dissertation submitted to the Graduate School of Yale University by the senior author as part requirement for the degree of Doctor of Philosophy.

Levine's eosin-methylene-blue agar was selected as the most satisfactory plating medium for the enumeration and differentiation of the coliform bacteria. The medium was plated with appropriate dilutions of intestinal contents or fecal suspensions, 0.5 or 1.0 ml. of the diluted material being used. The seeded plates were dried under sterile clay tops, then incubated for 48 hours at 37°C. and examined for coliform organisms.

A blood-agar medium—beef-infusion base containing 1% neopeptone and 3% defibrinated beef blood—was used for the cultivation of enterococci. Inoculation and incubation of the plates were carried out in the same manner as with the eosin-methylene-blue agar.

The deep-tube cysteine soft-agar medium of Valley (1929) minus the carbohydrate was chosen for the study of anaerobes, because of the ease and rapidity with which the examination could be carried out. The presence of sporulating anaerobes was determined by inoculating 1.0 ml. of the original suspension into the melted soft-agar tubes, heating the tubes at 80°C. for ten minutes, and incubating at 37°C. in an anaerobic jar. The tubes were examined at the end of three days, and again after one week's incubation.

Kulp's tomato agar medium, as modified by Rettger, Levy, Weinstein and Weiss (1935), gave the highest colony counts of all of the media used. The Petri plates were prepared from 1:10 to 1:1,000 dilutions of the test material. Duplicate sets of tomato agar plates were made, one was incubated under increased carbon dioxide tension and the other anaerobically, the method of Weiss and Spaulding (1937) being employed.

The sacrificed animals were placed on an operating board and the ventral surface of the animal sponged with 5% liquor cresolis compositus. The skin was cut along the midline and pinned back; the abdomen was opened and sections of the intestine clamped off with sterile hemostats. The segments were removed, one at a time, and the contents stripped into sterile Petri dishes.

In every instance the segment labeled "duodenum-jejunum" in this report was a section five inches long taken ten inches posterior to the stomach; the "ileum" was the four-to-eight-inch stretch of gut lying immediately anterior to the ileocecal valve; the segment from the colon was usually removed from the large intestine at a point three to four inches below the cecum; and the sample from the "rectum" was the most posteriorly formed fecal mass. The stomach and cecum were opened in approximately the mid portion, and some of the contents removed with a sterile pipette.

The pH of the stomach and intestinal contents was determined routinely by the colorimetric method, using brom-thymol-blue as the indicator, and known buffer solutions. Once in each experiment the pH determinations were checked against readings obtained with a Beckman glass electrode pH meter.

A loop of material from the original suspensions was spread on a slide and stained by a modified gram technic. The data obtained by this method are presented under the heading "Direct Microscopic Examination."

The composition of the experimental diets was as follows:

1. Normal stock diet consisting of oats in unlimited amounts and one daily feeding of washed lettuce weighing from 40 to 50 grams.

2. The balanced scorbutic ration of Dann and Cowgill (1935) fed in unlimited amounts and augmented by 1.0 ml. of orange juice per 100 grams of body weight per day. The diet was supplemented with water.
3. Cracked lentils given in unlimited amounts and the usual portion of lettuce fed daily.
4. Vegetable diet consisting of mangel-wurzel, about 108 grams of root, supplemented with 40 to 50 grams of lettuce daily.
5. Starvation diet augmented only by water.

A technic was devised by means of which a fine catheter (number 5 or smaller), of the kind used in cystoscopy, could be introduced into the stomach of the guinea pig and the material injected by a syringe attached to the outer end of the catheter.

The flora of the stomach is undoubtedly influenced by the ingested food and its flora. Information was sought regarding the numbers and types of bacteria and fungi present in the normal stock diet of lettuce and oats. A summary is presented in table 1 of the data gathered over a period of two years during which the feeding experiments were conducted.

Examination of fecal flora

The fecal flora of animals is a variable which can be determined only by the examination of a large number of samples taken over a considerable period of time. For this reason the intestinal flora of the guinea pigs was followed by daily examination of fecal specimens over a period of one week.

Cultural studies. The viable bacterial counts varied greatly from animal to animal, and from day to day in the same animal; however, there was a general trend in the kinds and numbers of microorganisms present in the feces.

Coliforms appeared only rarely on the standard Levine eosin-methylene-blue agar plates. The total colony count on this medium varied between 380 and 2,800 per 100 mg. of specimen. As compared with those obtained from man or from dogs, the figures are insignificant; even if they represented *Escherichia coli* alone. The bacterial growth on the plates was for the most part composed of subtilis-like and actinomyces-like colonies. Gram-negative *non-lactose* fermenting organisms appeared to the extent of about 50% of the total count.

Inasmuch as the enterococci are found in relatively large numbers in the feces of man and the dog, enterococci were anticipated in the guinea pig. The blood agar plates were used to bring out this flora and other organisms that have a preference for blood-containing media. Here again the total colony counts were very low, ranging between 1,500 and 9,400 colonies per 100 mg. of feces. Cocci were not observed in any of the plates.

Yeasts and yeast-like organisms were often present in guinea pig feces, but, with rare exceptions, the plate counts never ran higher than 200,000 per 100 mg. sample. It is interesting to note, on the other hand, that in a few of the animals a yeast-like flora was present to the exclusion of nearly all bacteria; the observations on these animals were fairly consistent in several daily examinations.

Tomato agar plates incubated anaerobically and under increased carbon dioxide tension showed a high degree of uniformity in the colony types. A few of

what appeared to be grain types of lacto-bacilli were seen, but fully 90% of the flora usually manifested itself in the form of small rough colonies having an average diameter of 100 micra. The colony counts varied from 2.6 to 800 million per 100 mg. stool. A very unusual anaerobic coccus form was observed also on the tomato agar plates, namely *Sarcina maxima* of Smit (1933), or a closely related organism. This anaerobe was of exogenous origin, but in a few instances

TABLE 1
Microbic flora of lettuce and oats
Average counts per gram of test material

TEST MATERIAL	COLI-FORMS	COCCI	LACTO BACILLI	GRAM + SPORULATING RODS		
				Aerobic	Anaerobic	Sarcina
Lettuce T	0	0	0	1,000	0	0
Lettuce B	0	0	0	63,000	10	65
Oats	1,100	0	390	1,200	3	0

T = top, and B bottom of lettuce.

The time required for the passage of food through the intestine of the experimental animal was determined by using gelatin capsules containing 100 mg. of finely divided animal charcoal. One capsule along with a small leaf of lettuce was administered to each of 8 guinea pigs. One-half hour later the animals received the regular ration of lettuce. Stool specimens were collected at 2, 6 and 10-hour intervals.

TABLE 2
Time for passage of labeled food through the intestine of guinea pigs

TIME	GUINEA PIG								
	1	2	3	4	5	6	7	8	C
hrs									
2	—	—	—	—	—	—	—	—	—
6	—	+	+	+	++	+	+	++	—
10	++	++	++	++	++	++	++	++	—

No charcoal particles visible microscopically or macroscopically, —; microscopically visible, +; Feces visibly colored, ++.

C = control animal.

At the end of six hours two of the guinea pigs voided stools that were darkened visibly, and by the eleventh hour all of the animals has passed charcoal-containing feces. It was concluded from this that in guinea pigs receiving a stock diet the time for passage of labeled food is between six and ten hours.

it was definitely established in the guinea pigs' digestive tract, and was eliminated in very large numbers.

Sporulating anaerobes were present in such small numbers as to be practically negligible, namely 15 to 50 colonies per 100 mg. of test material. What appeared to be *Clostridium sporogenes* was the most common member seen.

Direct microscopic examination. This indicated a much higher bacterial population than did the plating method; however, it may be assumed that the cells

observed were not all viable, and that the direct cell counts to a large extent represent dead and partially autolysed bacteria. Many gram-positive and gram-negative rods were observed on the slides, but the gram-positive rods rarely appeared as solidly stained cells. Rod-shaped bacteria and coccus-like organisms were the predominant forms; fusiform and spiral-shaped rods were next in order of frequency; the remaining stained material on the slides was made up almost entirely of gram-negative debris.

Examination of stomach and intestinal flora

Cultural. The flora of different segments of the intestine of guinea pigs on diets of lettuce and oats, scorbutic ration, and vegetables was not markedly different in the lower intestine from that found in the feces of animals held on the stock diet of oats and lettuce. In the small intestine there was considerable variation in the numbers and types of bacteria present.

The stomach, duodenum-jejunum and ileum contained few if any coliform bacteria or enterococci. Yeast-like organisms were present in small numbers in some of the animals in each of the three groups maintained on the different diets. Gram-positive non-spore-forming rods were the predominating flora of the upper segments of the intestine when there were significant numbers of bacteria present. It must be pointed out, however, that in the stomach and duodenum-jejunum the gram-positive rods were, on the whole, members of the grain types of lactobacilli, and not the small gram-positive rough colony form encountered in the fecal flora in the previous experiments. One animal carried on the vegetable diet contained large numbers of the small colony fecal form in the stomach, but it is quite possible that this was due to reinfection, since none of the other animals showed large numbers in the stomach. In the ileum there was at times a distinct fecal flora, but usually there was a transition flora with an increase in the numbers of the small-colony-producing rough form of lactobacillus. The genus *Clostridium* was present in the small intestine and stomach in such small numbers as to be practically negligible.

In the cecum and large intestine of guinea pigs fed the stock diet and the scorbutic ration coliform organisms were found in two animals, while the others showed no coli-like flora in these sections in dilutions as low as 1:10. Enterococci were found in the cecum and large intestine of three of the animals kept on the scorbutic ration, varying in numbers between 10,000 and 10,000,000 colonies per 100 mg. of contents. The colonies appearing on the blood agar plates, exclusive of the cocci, were composed of gram-positive sporulating cells, gram-negative non-lactose-fermenting rods, lactobacilli and a few actinomyces-like rods.

In animals harboring yeast-like organisms these were distributed throughout the digestive tract. The total numbers of colonies counted on tomato agar plates were, however, fewer than 50,000 per 100 mg. sample, barring a few exceptions. One guinea pig subsisting on the stock diet had a predominant yeast flora at all levels to the point of exclusion of practically all bacteria.

In the lower intestinal segments of the guinea pig fed the stock diet, scorbutic

ration and vegetable diet, the rough lactobacillus-like form appeared in large numbers on the tomato agar plates, namely from 10 to 50 million colonies per 100 mg. of intestinal contents. This organism constituted 90% of the total cultivable flora in the lower segments of the intestine, which is in strong contrast to the findings in the stomach and small intestine, the flora of which was definitely related to the flora of the feed.

Little if any variation in the numbers of the anaerobic sporulating organisms was noted in the animals held on different diets. When present in the intestine at all, the spore-forming anaerobes were cultivated in numbers fewer than 100 colonies per 100 mg. specimen.

Direct microscopic examination

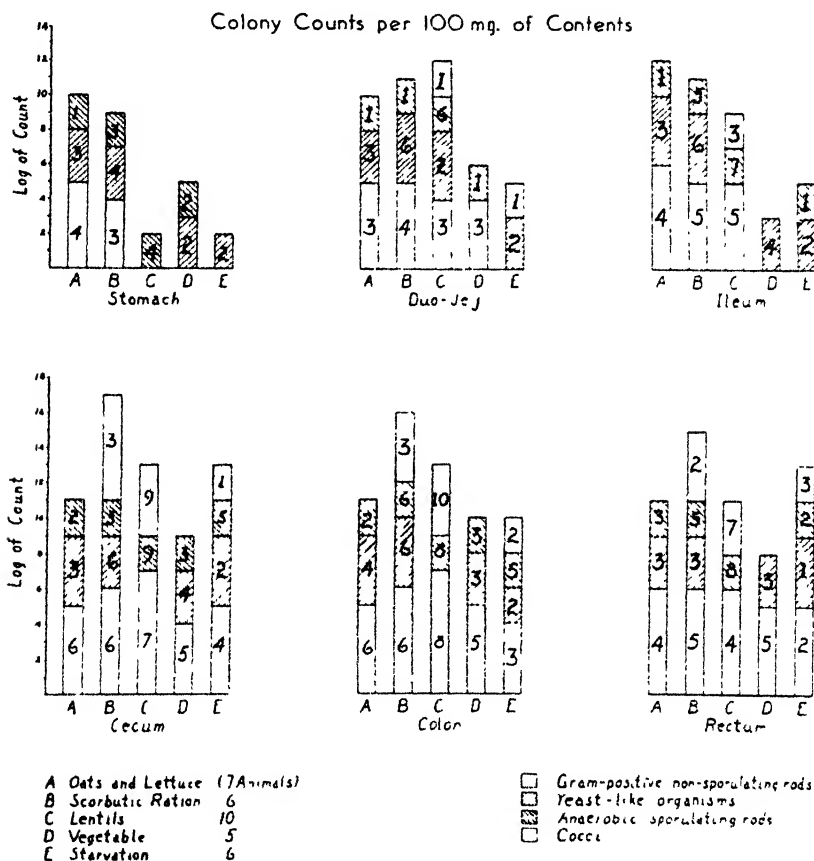
Gram-positive rods and cocci formed the predominating flora of the upper digestive tract. In many of the stained preparations the cells appeared to be undergoing autolysis with varying degrees of swelling and granulation. The guinea pigs fed the vegetable diet differed from the others in that the predominating flora was made up of gram-positive and gram-negative cocci in all of the segments studied. The animals fed the stock diet and the scorbutic ration yielded gram-positive rods and cocci in almost equal numbers, in some instances the rods were less numerous than the cocci. In material from the lower segments gram-negative rods, spirilla and fusiform bacteria occurred frequently. The total number of organisms appeared to be greatest in the colon.

In this investigation lentils were found to be the highest protein containing food that was acceptable to the guinea pigs. The fecal flora at the beginning of the lentil feeding experiment and at the end of one week had not changed from that of normal guinea pigs on the stock diet of oats and lettuce. At the end of two weeks several marked differences were noted. The color of the feces was altered from a greenish brown to almost a "pure" black; the consistency changed from that of a well-formed easily crushed stool to one that was very sticky and in some cases without form, and there was a definite "fecal" odor to the feces.

In the lentil feeding experiment the most striking change in the viable intestinal flora of the guinea pigs occurred in the numbers of cocci. In the stomach and upper stretch of intestine only one animal showed any cocci, while in the cecum and large intestine relatively high colony counts of cocci were obtained, varying from 5,000 to 20 million colonies per 100 mg. of test material. The rough (fecal type) gram-positive small-colony-producing bacterium was observed in the small intestine in relatively small numbers, with the exception of one guinea pig. In the cecum and large intestine this lactobacillus was apparently suppressed in six animals, and was present in the usual numbers in four. In the summary of results (see chart) it will be noted that from two to five animals failed to yield any gram-positive non-sporulating rods in the segments under discussion. At the same time there were relatively large numbers of cocci present which produced a zone of inhibition for the small colony form.

The direct microscopic examination of the stomach and intestinal contents

of guinea pigs kept on a diet of lentils showed the flora to be predominantly gram-positive rods and cocci. The frequency of occurrence was greatest in the lower segments, cecum and colon.



STOMACH AND INTESTINAL FLORA OF GUINEA PIGS ON VARIOUS DIETS

This chart is an attempt to present in one series of graphs the distribution of four different groups of bacteria observed in the digestive tract. The figures in the columns indicate the number of guinea pigs yielding the bacterial counts plotted. For example, the stomach bar "A" shows that of seven animals fed on oats and lettuce diet four had 100,000 gram-positive non-sporulating rods per 100 mg. of test material in the stomach, three had 1,000 yeasts; and one of the seven had 100 spore-forming anaerobes.

The original tabulated results are too extensive to include in this report. For a more complete summary, readers are referred to the tables in the senior author's Ph.D. dissertation, on file in the Sterling Library of Yale University.

The starvation diet was maintained for four days; examination at the end of that period showed the intestinal flora to be unchanged as to kinds of micro-organisms present, but greatly reduced in numbers in a majority of the animals used. The gram-positive small-colony (fecal) form was affected more than the other types, being largely or entirely suppressed.

Direct microscopic examination of stained smears of intestinal contents of

the starvation-diet guinea pigs revealed cocci as the predominating flora of the cecum and large intestine. The upper segments appeared to be relatively free from bacteria.

BRIEF SUMMARY

The data reported here show that the intestinal flora of the guinea pigs was relatively simple and contained few bacterial types. The predominating cultivable flora was a lactobacillus apparently closely related to *Lactobacillus acidophilus* and *L. bifidus*. It constituted about 80% of the total cultivable flora. Yeast-like organisms were present in the intestine, irrespective of the diet, and in some instances they were present to the exclusion of practically all bacteria.

It will be noted that three common fecal organisms, the coliforms, enterococci and sporulating anaerobes, were not demonstrable as part of the cultivable intestinal flora. These organisms, if they were present at all, were there presumably as exogenous organisms, and not as part of the guinea pig intestinal flora.

Among the organisms observed in the guinea pigs two are of particular interest. They are (1) the small gram-positive non-sporulating bacillary form of the intestinal lactobacillus type, and (2) the large anaerobic highly aerogenic sarcina. Both frequently appeared in the digestive tract in large numbers, and gave definite indications of having undergone extensive multiplication there. As the former is obviously a member of the *Lactobacillus* genus it is referred to here as an intestinal lactobacillus.

THE INTESTINAL LACTOBACILLUS

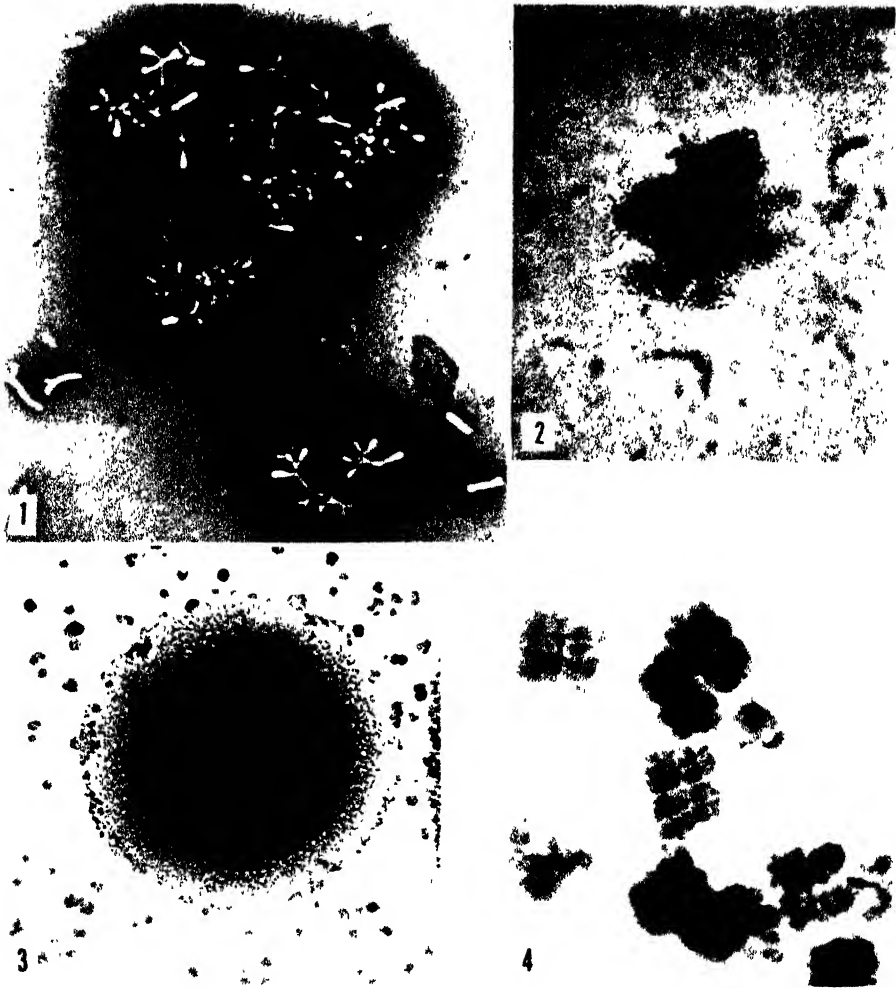
This organism was isolated from the feces and intestinal contents of guinea pigs by plating on tomato agar under either anaerobic or microaerophilic conditions. After repeated transfers in semi-solid tomato cystein agar, cultures could be maintained indefinitely in this medium, and successful cultivation could be brought about in plain tomato broth.

On solid tomato agar the cell morphology was usually that of a small rod 0.5–0.8 by 2.5–8.0 micra in size. The cells occurred single or in pairs, rarely in chains. Gram-stained preparations showed more or less granulation. Varying degrees of pleomorphism were exhibited ranging from single cell to multi-branched forms (see fig. 1). Capsules and motility were not observed.

Recently isolated strains produced deep pin-point colonies in tomato agar within 24–36 hours which under the low-power lens appeared circular to elliptical. Colonies which reached the surface of the agar revealed a more or less dense structure from which hair-like filaments extended into the medium (see fig. 2). The colonies increased in size under favorable conditions, but remained delicate and never attained a diameter of more than 300 micra.

Established strains produced slight turbidity in tomato broth within 48 hours; this was followed soon by sedimentation. In tomato broth medium containing 10 per cent gelatin and 1.5 per cent cystein hydrochloride, and inoculated liberally with young cells, a uniform turbidity developed within 24 hours at 37°C.;

by the end of 72 hours incubation there was abundant growth which settled out and occupied one third of the shake tube column. The gelatin was not liquefied by the organism.



Figs 1 to 4

Upper left. Showing cell morphology of the intestinal lactobacillus in tomato broth culture incubated at 37°C for 72 hours. $\times 600$ Nigrosin stain.

Upper right. Typical deep colony of the lactobacillus after reaching surface of medium, tomato agar incubated in closed chamber with added CO_2 at 37°C for 72 hours. $\times 100$

Lower left. Surface colony of anaerobic sarcina on tomato agar after 18 hours incubation at 37°C. $\times 20$. Note broken agar caused by escaping gas.

Lower right. Showing cell morphology of the anaerobic sarcina. Tomato broth culture incubated 6 hours at 37°C. $\times 1000$ Nigrosin stain.

No visible growth took place at 18, 22 and 50°C. At 43° only five of the strains appeared to develop. Good growth occurred at 37° within 48 hours.

Hydrogen ion concentration tolerance was determined in tomato broth ad-

justed to various pH values with the aid of the Beckman glass electrode. The lower limits of growth lay between 4.5 and 5.0. The optimum growth range was pH 6.6-6.8. The upper limits of visible growth were between pH 7.5 and 8.0. The final H-ion concentration attained in 72 hours in soft cystein tomato agar was 4.5-5.0, except for a few strains which brought the pH down to 4.2.

Growth is favored strongly by anaerobic or micro-aerophilic conditions. Total colony counts are very much higher in the presence of added CO₂ or under anaerobic conditions than in plates that are incubated aerobically.

None of the strains exhibited hemolytic properties. Litmus milk was strongly acidified and clotted by only 3 strains. The same strains also reduced the litmus.

All of the guinea pig strains fermented glucose, levulose, maltose and sucrose, without gas production, and all but two attacked lactose and raffinose. However, classification on the basis of fermentation of carbohydrates was not satisfactory, because there was considerable variation in the powers of some of the strains to attack carbohydrates consistently. The total titratable acidity in tomato broth varied between 4.90 and 10.00 ml. N/10 acidity per 10 ml. of the medium. Uncompleted experiments indicated that the amounts of fixed and volatile acids did not differ materially from those produced by known *L. acidophilus* of human origin.

Attempts to relate the guinea pig lactobacillus to other lactobacilli by means of agglutination and precipitin reactions proved unsatisfactory.

Catalase production could not be demonstrated in any of the strains. Tests made at the same time with known strains of *Propionibacter* gave positive results.

THE ANAEROBIC SARCINA ISOLATED FROM THE FECES OF GUINEA PIGS

This organism is given some prominence here, first, because it often occurs in large numbers in the intestine of guinea pigs, and, second, because it is an unusual type of *Sarcina*, with which many bacteriologists do not appear to be familiar. Indeed, Bergey's Manual (1939) lists Goodsir's sarcina as an aerobic coccus, the cells measuring 2.5 micra in diameter. The real nature of Goodsir's *Sarcina* was not fully revealed until Beijerinck (1911) isolated it or a closely related packet form by employing acidified beer wort in flasks which were filled to the mouth and stoppered, to create anaerobic conditions. He succeeded in this manner in separating it from aerobic sarcina forms with which it is so commonly associated, particularly in the stomach.

Smit (1933) claimed that he had isolated and described the true *Sarcina ventriculi* of Goodsir (1842), while engaged in a comprehensive study of anaerobic sarcinae. He reported it to be a very large anaerobic packet form; also a powerful gas producer possessing high acid tolerance. Smit's claims were recognized by Weinberg and his associates (1937). Smit also isolated and described an anaerobic sarcina which was on the whole quite similar to *Sarcina ventriculi*. The two differed, however, in one important respect. The new organism, to which he applied the name, *Sarcina maxima*, was a strong producer of butyric acid, whereas this substance did not constitute one of the fermentation products of *S. ventriculi*.

The organism found by the present authors in the intestine and feces of guinea pigs bore a striking resemblance to Smit's *S. maxima*, and is regarded by them as being identical with it or a close variant of it. It is referred to in this paper, therefore, as *Sarcina maxima*.

The organism is a large gram-positive non-sporulating anaerobic coccus which develops in characteristic packet form. It was first observed on tomato agar plates which had been inoculated with guinea pig feces. Attempts to isolate it failed until an acidified tomato broth and strictly anaerobic conditions were employed. By adjusting the reaction to pH 4.0 the small colony-forming lactobacillus was inhibited.

Deep colonies of a pure culture usually raised the agar from the bottom of the Petri dish. A striking characteristic of the deep colonies was their angular appearance. The larger colonies attained a size of about two millimeters in diameter during twelve hours' incubation at 37°C.

Surface colonies produced under the same conditions were 2-5 millimeters in diameter; they were round and had a raised surface and an internal microscopic structure resembling that of a yeast colony (see fig. 3). When magnified 100 times the large packets were seen to be made up of units of 8 cells. There was very little or no chromogenesis on tomato agar plates. A definite odor of butyric acid was emitted from the anerobic jars at the time they were opened.

Tomato broth growth was obtained readily when large amounts of inoculum from broth or agar cultures were employed. Growth was vigorous by the end of 6 hours incubation at 37°C. It was typified by marked gas evolution throughout the tube, the bubbles being released apparently from the heavy sediment in the bottom of the tube. At the peak of growth the sediment was churned and carried up through the fluid by the gas bubbles. A "head" of foam developed on the surface of the tomato broth after six and up to 12 hours of incubation.

The cell morphology and orientation presented striking microscopic pictures. The cells were 3.3-4.0 micra in diameter, and when alone appeared spherical. In packets of 8 cells or more the adjacent cell surfaces were flattened and seemingly bound together firmly (see fig. 4). The most commonly observed packets were made up of 32 cells. Capsules could not be demonstrated. During the first 6 hours of incubation the cells were strongly gram-positive. After 12 hours practically all of the cells were negative by the gram stain. Spore formation was at no time apparent.

Growth took place at 20, 30 and 37°C., but not at 50. It was most vigorous at 37°C. The lower limit of pH which permitted growth was 4.0, and the upper 8.5. Reactions on the acid side of neutral were more favorable than on the alkaline.

The organism required added carbon dioxide, and almost complete anaerobiosis. Bacto-peptone and "savita" yeast extract were adequate sources of nitrogen. A fermentable carbohydrate was necessary for growth. Slight growth took place when 0.1 per cent glucose was present. For luxuriant development 2.0 per cent was necessary. The sarcina utilized glucose, levulose, galactose, maltose and lactose, but not mannose, xylose and raffinose.

A comparison of this anaerobic coccus with Smit's *Sarcina maxima* reveals a close similarity, except in the following respects: *S. maxima* has a pH tolerance as low as 0.8, while that of the guinea pig organism is 4.0. The former was found in soil and grains and, according to Smit, could not be isolated from the feces of laboratory animals, whereas the latter was not only present in various guinea pigs examined, but could implant itself for varying periods of time in the intestine of guinea pigs.

From the numerous characters which the two *Sarcina* forms have in common, in spite of the few differences cited here, we may assume the guinea pig coccus to be a variant of *Sarcina maxima*. It is proposed here to apply to it the name, *Sarcina maxima* Smit (variant).

SUMMARY AND CONCLUSIONS

This investigation was undertaken to determine the normal flora of guinea pigs subsisting on a stock diet of lettuce and oats, and to study the effect of changes of ration upon the intestinal flora. Five different diets are reported here.

The normal flora of the guinea pig intestine was found to be relatively simple, consisting of a small number of bacterial types and of yeast-like microorganisms. The predominating organism was a small gram-positive, non-sporulating rod form of the *Lactobacillus* genus. It constituted approximately 80% of the total cultivable flora. The other 20% consisted essentially of what appeared to be yeasts, soil and air bacteria. Throughout the course of the investigation, with one exception, other commonly occurring intestinal bacteria, particularly *Escherichia coli* and the enterococci, were absent, or were present in very small numbers only.

The predominant organism bears a close resemblance to *Lactobacillus acidophilus* of human origin, particularly in its preference for microaerobic and anaerobic conditions, cell and colony form, and nutritional and temperature requirements. It produces smaller and more delicate colonies on appropriate media, and in general appears to be a less hardy lactobacillus than *L. acidophilus* of rat origin. There is every reason to regard it as *L. acidophilus*; for the sake of convenience and host reference the name, *L. acidophilus*, variety *caviae* might well be applied to it.

A second organism of particular interest in the present study was a large anaerobic sarcina form which was highly aerogenic. It occurred in some of the guinea pigs in large numbers and was apparently capable of implanting itself in the intestine of guinea pigs. This organism resembles Smit's *Sarcina maxima* to a very large extent, and should perhaps be designated as such, or a variant thereof.

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BACTERIAL MORPHOLOGY AS SHOWN BY THE ELECTRON MICROSCOPE

V. TREPONEMA PALLIDUM, T. MACRODENTIIUM AND T. MICRODENTIIUM

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The treponemata present morphological features of singular interest which have been the subjects of a multiplicity of interpretations over a period of more than thirty-five years. Electron micrographs permit the examination of these structures with greater precision, and hence can contribute, within limits, to their interpretation.

An electron microscopic study of *Treponema pallidum* directly from syphilitic lesions has recently been published by Wile, Picard and Kearny (1942). Morton and Anderson (1942) have examined the cultured Nichols-Hough strain of *T. pallidum*. A previous paper by the present authors (Mudd, Polevitzky and Anderson, 1942), and the present article include electron micrographs of the cultured Noguchi, Nichols-Hough, Kroó and Reiter strains of *T. pallidum* and of *Treponema microdentium* and *Treponema macrodentium*. A picture of treponemata of the virulent Nichols-Hough strain from a testicular lesion in a rabbit is also included.

From these studies it is clear that the inner protoplasm of treponemata, like that of all bacteria studied, is enclosed in a definite cell-wall. In the case of the treponemata this cell-wall is a sheath or periplast of extreme delicacy, which may be continuous between incompletely divided spirochetal cells and may extend beyond the cell protoplasm as a terminal filament. Flagella are frequently or regularly present; these appear in groups either along the sides or near the ends of the spirochetal cells. Dense granules in certain preparations are present within the cell protoplasm. Finally, and most challenging to further investigation, dense spheroidal bodies are often found attached to the treponemata by short stalks or free in the medium.

The strains studied were all received from Miss Clara C. Kast, Research Institute of Cutaneous Medicine, Philadelphia; these strains and the media used for their cultivation have been described by Kast and Kolmer (1940). The cultures of *T. pallidum* were in the cysteine medium described, and those of *T. macrodentium* and of *T. microdentium* in 0.5 per cent glucose hormone broth with 10 per cent ascitic fluid to which a small piece of sterile neutralized kidney tissue had been added. The cultures were vaseline sealed and incubated 2 or 3 days or longer.

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For electron microscopic study a sample of the culture was removed by a capillary pipette and mixed with sterile distilled water; the suspension was centrifugalized at about 1500 r.p.m. for 20 minutes. The sediment was resuspended in distilled water and recentrifugalized. The process was repeated a third time. The final sediment was resuspended in distilled water and a droplet of the suspension immediately dried on the collodion mount as usual for introduction in the electron microscope. It is probable that the size of the cells and their spirals may have been altered by the manipulations described; these features are therefore not discussed in the text below.

The cell-wall. The cell-wall ("Periplast," Schaudinn, 1907; "périplasmic," Manouélian, 1940) in some preparations encloses the protoplasm so closely that it cannot be distinguished separately. In other preparations, however, the delicate cell-wall unites daughter spirochetes which have not completed their transverse division, or extends beyond the protoplasm at the end of a spirochetal cell (figs. 1 and 2); other pictures show the protoplasm shrunken away from the surrounding cell-wall (fig. 3); or the cell-wall may remain as a "ghost" of a cytolysed cell from which the protoplasm has escaped (fig. 7). The periplast is obviously not an "undulating membrane" as believed by Schaudinn and other early observers who regarded *Spirochaeta pallida* as a protozoön.

According to Noguchi (1928), "The cell-body consists of a spiral, elastic axial filament, a layer of protoplasm of varying thickness around the filament and a delicate flexible membrane covering the whole body." In none of the electron pictures in this or earlier studies have we found evidence of the existence of such an elastic axial filament as a differentiated structure. The "axial filament" connecting adjacent cells in Noguchi's figures we believe to have been the delicate cell-wall joining incompletely divided cells. Noguchi believed the elastic axial filament to be requisite to explain motility; he was not, however, aware of the existence of flagella, which are demonstrated in this and preceding papers (Morton and Anderson, 1942; Mudd, Polevitzky and Anderson, 1942).

Flagella. Flagella are seen in electron micrographs of all of the strains of *T. pallidum* (figs. 1, 2, 3, 4 and 6) and the strain of *T. macrodentium* studied (fig. 7). We have not been able to demonstrate flagella on our pictures of *T. microdentium*. The flagella are lophotrichate, in many of the pictures occurring in groups of four, situated either near the end or along the sides of the spirochetal cell. The flagella are from 14 to 17 μ in diameter. The uniformity of the flagellar diameters of the several strains of treponemata (when calculated to the same magnification) is noteworthy.

Under *Treponema pallidum* in Bergey's Manual (1939) the plain statement "flagella absent" is made, but the genus *Treponema* is described as "with or without flagelliform tapering ends." Many of the early descriptions of stained preparations of spirochetes (Herxheimer and Löser, 1905; Schaudinn, 1907; Uhlenhuth and Haendel, 1907; Noguchi, 1912a) describe a terminal flagellum at one or both ends of the spirochetal cell. The flagella of typhoid bacilli in locomotion are plaited together to form single spiral filaments (Pijper, 1941); these are visible in the dark-field microscope with the special equipment of

Pijper, but not with ordinary equipment. The flagella of a lophotrichate water bacterium recently isolated by Hutchinson and McCracken (1942) form in loco-

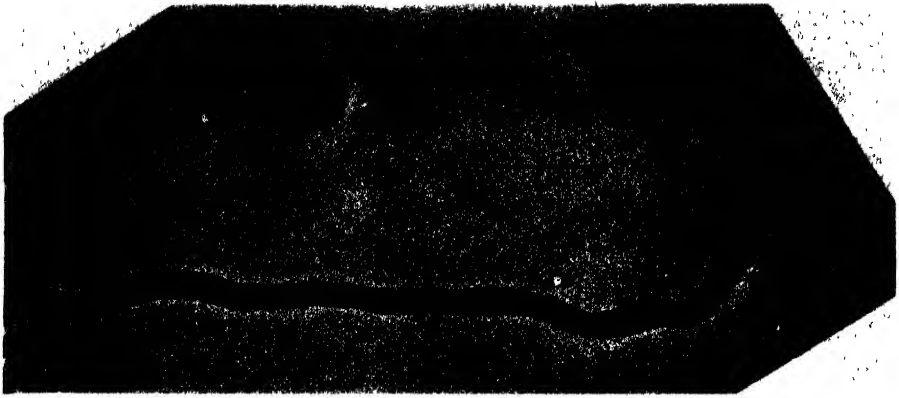


FIG. 1. *T. PALLIDUM* (REITER STRAIN). $\times 14,000$

The segment of a spirochetal cell below shows a terminal extension of the cell-wall or periplast beyond the protoplasm; a tuft of flagella arises from the side of the cell. The upper segment shows a terminal "end-body"; a somewhat vague granule within the protoplasm is shown proximal to the "end-body."

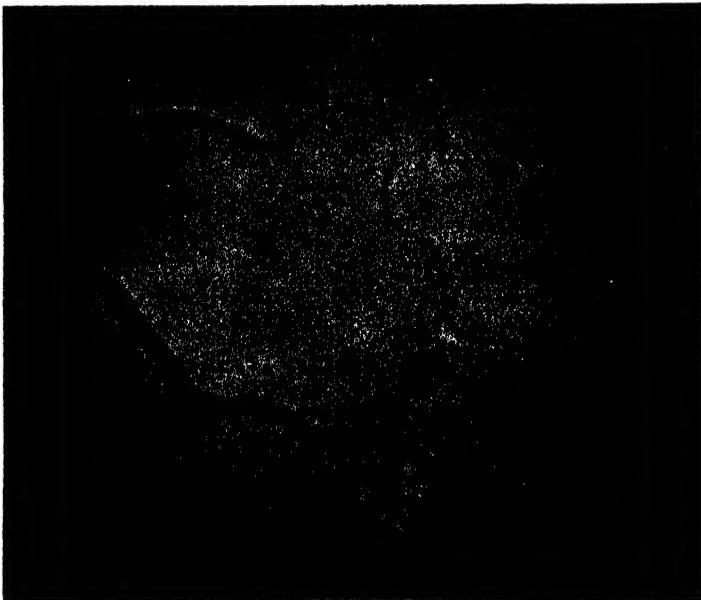


FIG. 2. *T. PALLIDUM* (NICHOLS-HOUGH STRAIN). $\times 14,500$

The cell-wall or periplast is continued beyond the protoplasm of the spirochetal cell as an end-filament. Flagella are seen arising from the side of the cell.

motion spirally-wound filaments attached to each end of the bacterium, which may be seen with ordinary dark-field equipment; each filament appears in the dark-field as single, but each may be resolved with the electron microscope into



FIG. 3. *T. PALLIDUM* (KROÓ STRAIN). $\times 27,000$

The spirochetetal cells in this preparation appear to be more or less cytolyzed. To the left a tuft of four distinct flagella are seen. Above to the right is a segment of a spirochetetal cell in which the dark inner protoplasm is shrunken away from the cell-wall surrounding it. Above is what appears to be a tuft of flagella plaited together.

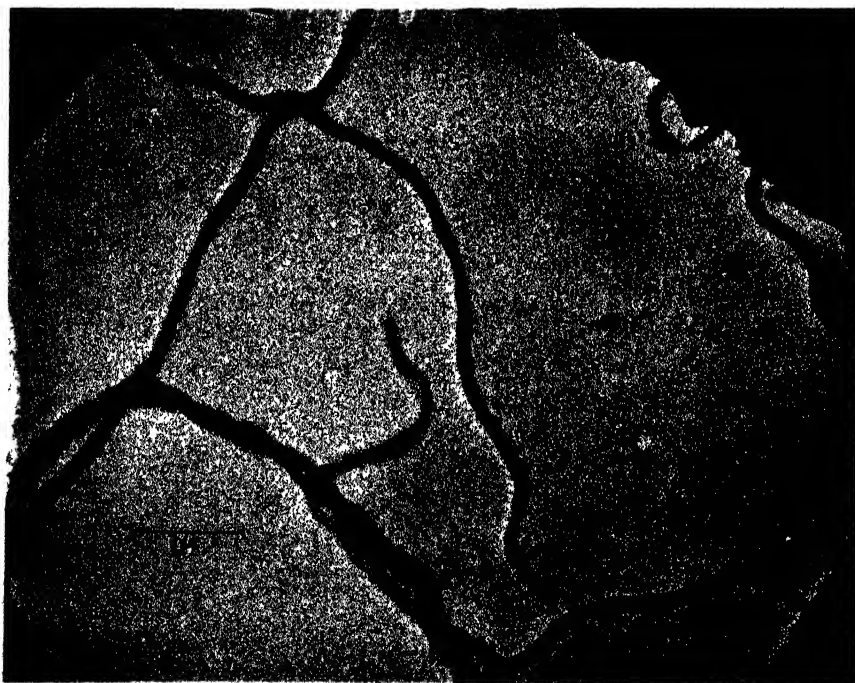


FIG. 4. *T. PALLIDUM* (NICHOLS-HOUGH STRAIN). $\times 14,000$

Intertwined spirochetetal cells. Granules, 40 to 90 $m\mu$ in diameter, are clearly shown within the protoplasm. One tuft of four flagella is clearly and other tufts are somewhat vaguely seen.



FIG. 5. *T. PALLIDUM* (REITER STRAIN). $\times 14,000$

The protoplasm is uneven in density and may well have been in process of degeneration. A dense spheroidal body, 290 to 400 m μ in diameter, is attached to the spirochetal cell near its left end (cf. Wile, Picard and Kearny, 1942, figs. 1, 2, 4; and Morton and Anderson, 1942, figs. 3, 6, 7 and 8).

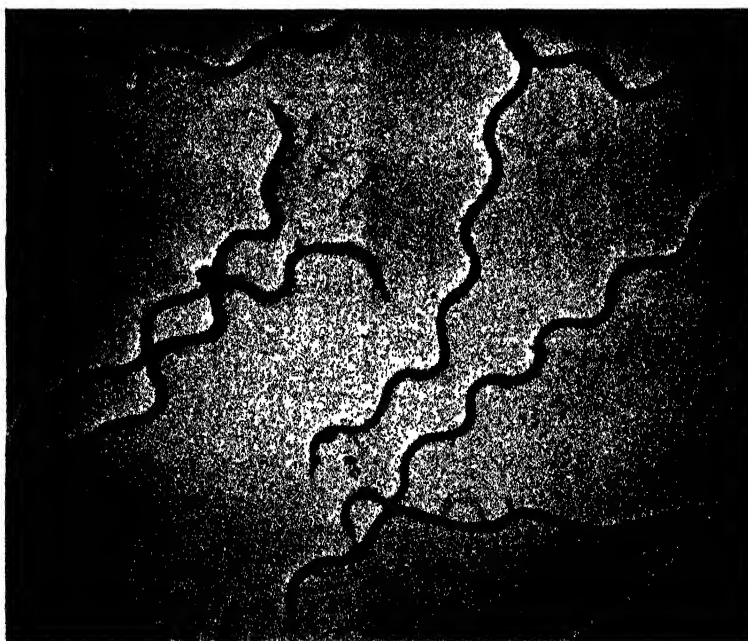


FIG. 6. *T. PALLIDUM* (VIRULENT NICHOLS-HOUGH STRAIN FROM RABBIT SYPHILOMA). $\times 12,500$

A dense, spheroidal body about 155 m μ in diameter is attached to one spirochete; a tuft of flagella appears on another cell.



FIG. 7. *T. MACRODENTUM*. $\times 13,000$

Two intact cells and one "ghost," or cell-wall empty of protoplasm, are seen. Two tufts of flagella remain attached to the empty periplast of the "ghost."



FIG. 8. *T. MICRODENTUM*. $\times 14,500$

Intertwined spirochetal cells simulate the appearance of longitudinal division. The point where one cell crosses over the other is indicated by an arrow. A dense spheroidal body, about 170μ in diameter is similarly indicated.

multiple flagella. It is possible that the flagella of spirochetes, similarly plaited together, may have been seen in stained preparations by the early observers; it is also likely, however, that terminal extensions of the periplast may have been mistaken for flagella in some instances. Restudy of the locomotion of spirochetes with equipment of the excellence of Pijper's would be of interest.

Granules within the protoplasm. Granules within the spirochetal protoplasm are seen in figs. 1 and 4 (and in fig. 4A, Mudd, Polevitzky and Anderson, 1942). These appear to be dense spheres 40 to 90 m μ in diameter; they resemble granules seen within the protoplasm in micrographs of a variety of bacteria (Mudd, Polevitzky and Anderson, 1942; Knaysi and Mudd, 1943). In the absence of adequate cytological data on these granules in spirochetes we would rather not attempt to interpret them. Granules within the protoplasm were shown in a drawing of a stained spirochete by Herxheimer (1905) and distinguished by him from the spheroidal bodies next to be described.

"Granules spirochétogènes." Dense, irregularly spheroidal bodies may be attached to the spirochetal cells, frequently near the ends of the cells. Some of these dense bodies are closely applied to the sides of the spirochetes; others are attached to the side of the spirochetal cell by short stalks, and others are found free near the spirochetes. Such dense bodies are shown in figs. 5 and 6, in Morton and Anderson (1942) figs. 3, 6, 7 and 8, and in Wile, Picard and Kearny (1942) figs. 1, 2 and 4. These bodies have been described by a long series of investigators; the terms frequently applied to them, "Knospen" or "buds," (Meirowsky, 1913, 1925); "spore-like spherical bodies," (Noguchi, 1912c); "granules spirochétogènes," (Manouélian, 1935, 1940), express implicitly the interpretation often explicitly made that these are asexual reproductive bodies. The very impressive accumulation of evidence supporting this interpretation has been reviewed by Meirowsky (1930) and Ingraham (1932), and more recently by Manouélian (1940).

The extreme monomorphic view has, however, not been without its proponents. Thus Bessemans (1938) writes: "The so-called atypical forms of *T. pallidum* are only fragmented and altered organisms. . . . The organism causing syphilis is and remains a treponeme. In other words its morphologic appearance is always the same, barring the slight variations in its dimensions and motility which have been described."

The spheroidal bodies shown in the electron micrographs cited we certainly do not believe can reasonably be interpreted as degeneration products. They are definite and characteristic bodies originating from the spirochetal cell.² Morphologically they resemble endospores (Mudd, Polevitzky, Anderson and Chambers, 1941) in their high density relative to the protoplasm of the vegetative cells; the spheroidal bodies of spirochetes are of course smaller than endospores and differ from them also in their positions at the sides of the vegetative cells. The spheroidal bodies are similar in size to the reproductive granules of the micro-organisms of the pleuropneumonia group (Ledingham, 1933; Sabin,

² These bodies were also observed by the senior author many years ago in studying culture spirochetes in oil-water interfaces.

1941). Pleuropneumonia-like micro-organisms have recently been described by Dienes (1942) as associated, he believes as variants, with a number of kinds of bacteria. In their position at the sides of vegetative cells, at times on little stalks, the spirochetal spheroidal bodies recall the conidia and chlamydospores of higher fungi (Weidman, 1933, 1939). Whatever the taxonomic relationships of these spirochetal spheroidal bodies may be, the important fact is that they are not artefacts, impurities from the medium, or products of degeneration; hence they must have a positive significance, and it is difficult to understand, considering all the evidence, what this significance might be if not that of asexual reproductive bodies.

"End-bodies." A peculiar structure which appears to be formed by a rolled-up end of a spirochetal cell is shown in figure 1. Such structures have been observed by many students of the spirochetes. Meirowsky (1925) identified them with the spore-like bodies we have described above; this appears, however, to have been a misinterpretation. The most exact descriptions of such "corpuscules arrondis" we have found are given by Manouélian (1935, 1940) who regarded them as probably involutionary.³

Division. Schaudinn (1907) and many other early investigators believed that cell-division among the treponemata was longitudinal. Noguchi (1912b) in his earlier work definitely supported this view; later, however (1917), he wrote: "It is highly probable that the usual mode of division in culture is transverse, although the possibility of longitudinal division cannot be excluded." Finally Noguchi (1928) simply described division of the treponemata as transverse.

Figure 8 shows the type of morphologic appearance upon which the interpretation of longitudinal division was based (the light microscopes used did not resolve the separate but intertwined treponemal cells). The point at which one treponemal cell of an intertwined pair crosses over the other is indicated by an arrow.⁴ Division is now generally agreed to be transverse (Manouélian, 1940).

A second study on *T. pallidum* from human lesions appeared after galley proof had been read on the present communication (Wile, U. J. and Kearney, E. B., The Morphology of *Treponema Pallidum* in the Electron Microscope. Demonstration of Flagella. J. Amer. Med. Asso., 1943, **122**, 167).

SUMMARY

Electron micrographs of the Nichols-Hough, Kroó and Reiter cultured strains of *Treponema pallidum*, of treponemes of the virulent Nichols-Hough strain from a rabbit syphiloma, and of cultured strains of *Treponema macrodentium* and *Treponema microdentium* are presented and the morphology of the treponemal cells described.

A delicate cell-wall or periplast encloses the inner protoplasm of treponemata;

³ A consultant mycologist has suggested, in view of the spiral arrangement of this structure and the fact that granules appear within it, a possible relationship to an abortive perithecium such as appears in the ascomycetes.

⁴ This is an interesting illustration of the fact that the depth of focus with the electron microscope is considerably in excess of the diameter of such objects as bacteria (Anderson, 1942).

this periplast may connect adjoining cells until transverse cell division is completed; thereafter it may extend beyond the cell protoplasm as a terminal filament. No evidence of a differentiated axial filament within the protoplasm is found.

Flagella, often in groups of four, are found along the sides or near the ends of the cells of *T. pallidum* and *T. macrodentium*.

Dense granules, 40 to 90 m μ in diameter are often found within the spirochetal protoplasm.

Irregularly spheroidal, dense bodies, 150 to 500 m μ in diameter, are often found attached to the spirochetal cell, frequently near the end; such a dense body may be in close apposition to the outside of the spirochetal cell-wall or may be connected to it by a short stalk. The evidence concerning these bodies seems to support the interpretation that they are asexual reproductive bodies.

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OPTIMUM AND LIMITING TEMPERATURES FOR THE GROWTH OF THE PLAGUE BACILLUS IN BROTH

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It has become customary to grow pathogenic organisms in the laboratory at 37°C. This practice seems to be based on the belief that those organisms "which have adapted themselves to a saprophytic or parasitic life in relation with warm-blooded animals have an optimal temperature round 37°C. to 38°C." (Andrewes, 1930). This belief does not appear to be based on any exact measurement of optimum growth temperatures for the various organisms concerned. In the case of *Pasteurella pestis*, at least, it has long been recognized by most workers that it grows "poorly above 35°C." though there is still a wide difference of opinion as to the temperature at which optimum growth results. One of the present authors (Sokhey 1939b) had found the temperature for optimum growth in broth to be about 27°C. More recently Spicer (1940) has reported that, "the optimum temperature for the growth of a Type III pneumococcus strain was found to be 27°C."

In the case of *P. pestis* early workers, quoted by Albrecht and Gohn (1900), found the optimum growth to result at 37°C.; later workers, as reported in standard textbooks, put the optimum growth temperature at 25°C. to 30°C., though Topley and Wilson (1936) state, "growth, however, at both 24°C. and 37°C. is often as good as at 30°C." Recently Tumansky *et al.* (1935) made a comparative study of the growth of *P. pestis* and *Pasteurella pseudotuberculosis* Pfeiffer, at nine different temperatures beginning with 0°C. and ending with 43°C. They found the optimum growth temperature of *P. pestis* to be 28°C. to 30°C. and the upper limit of growth at about 43°C. Regarding the lower limit of the growth they found that the organisms showed some growth even at 0°C.

It would appear that at least some of the differences in the reported observations are due to different media, solid or liquid, employed, and the number of organisms used as the inoculum by different workers. In a previous paper (Sokhey 1939b), it was shown that on nutrient agar plates, if 5,000 organisms or more were seeded per square centimeter of the surface, more profuse growth resulted at 27°C. than at 37.5°C., but if a smaller inoculum was used, i.e., 500 organisms per sq. cm. of the surface, no growth resulted at all at 27°C. while some growth still appeared at 37.5°C. after incubation for 48 hours. If blood agar was substituted for nutrient agar even with the smaller inoculum, 500 organisms per sq. cm. of surface, the number of colonies resulting both at 27°C. and 37.5°C., were equal in number, though the size of the colonies at 37.5°C. was smaller.

In the present paper results are given of an investigation carried out to de-

termine more precisely the optimum and limiting temperatures for the growth of *P. pestis* in nutrient broth.

TECHNIQUE

The optimum temperature for the growth of an organism depends on a variety of factors. The most important of these are: (1) composition of the medium, (2) number of organisms used as the inoculum, (3) the time chosen for observation, and (4) the criterion used for determining the optimum growth. Therefore, for a comparative study like this the conditions under which the experiment is conducted must be specified and kept constant.

Medium and its pH

The present study refers exclusively to the growth of the organism in nutrient broth. Basal Infusion Broth, Medium No. 748, Committee of A.P.H.A., was used (Levine and Schoenlein, 1930). Two different pH values of broth, 6.4 and 7.2, were used for reasons explained below. Broth for each determination was placed in 10 ml. quantities in test tubes with an internal diameter of 1.7 cm. Keeping the internal diameter of the tubes constant is important since it was found, as reported in a previous paper (Sokhey 1939c), that "a 48-hour growth of the plague bacillus in a liquid medium bore no relation to the total quantity as such of the medium nor to its surface area, but was directly proportional to the circumference of its surface area." Though subsequent work, which will be reported in another paper on the rate of growth of the organism, has shown that this statement is subject to a modification, the observation still remains true in its broad aspect and applies to the present study.

For the experiments reported in this paper the same batch of broth was used throughout for each of the two series of determinations. Large quantities of broth were made and kept in cold storage at 4°C. For the preparation of the inoculum, however, odd batches of broth with pH 6.8 were used.

Inoculum

Preliminary observations showed that as small an inoculum as 40 to 60 organisms sown in 10 ml. of nutrient broth, in our test tubes, was enough to give growth, but with this inoculum appreciable amount of growth resulted only after a very long period of incubation, about 8 days. A larger inoculum, say 3 to 4 million organisms, gave a good growth in 36 hours and could be relied upon to minimize the stationary (lag) period of growth, or rather the stationary periods of growth of *P. pestis*. In a subsequent paper we shall show that the plague bacillus when grown in a liquid medium has two stationary periods of growth. For our inocula 48-hour growths of the organism in nutrient broth were used. They were prepared in the following manner. A 2 mm. loopful of a culture on blood agar was inoculated into 10 ml. of nutrient broth in a test tube. The growth on the loop was carefully mixed with broth to obtain a tolerably uniform suspension and was incubated at 27°C. for 48 hours. A second

subculture was made by sowing 0.5 ml. of the first subculture into 9.5 ml. of broth in a test tube (1.7 cm. internal diameter). This was incubated in a vertical position for 48 hours at 27°C. The second subculture contained approximately 400 million organisms per ml. It was diluted to 1 in 10, and 0.1 ml. of this dilution was used as the inoculum for inoculating 9.9 ml. of the broth for each determination. Actual counts showed that the inocula on an average contained 3.46 million organisms.

Criterion of optimum growth

Crop yield after exactly 36 hours of incubation was used as the criterion of optimum growth. In our hands crop yield proved to be quite an effective criterion and its measurement was less laborious than the measurement of the rate of growth. The broth cultures were incubated in a vertical position and were specially protected against mechanical jars. The crop yield was estimated by the method of counting the number of viable plague organisms in broth cultures, described in a previous paper (Sokhey 1939c).

Strains

Two strains of *P. pestis*, 55/H and 145/Bit, were used, one for each of the two series of experiments reported. These strains were the first subcultures on blood agar made from primary culture of venous blood from severe septicaemic human cases. The virulence of the subcultures had been measured by the method described in a previous paper (Sokhey 1939e), and both strains were highly virulent, i.e., 5 to 10 organisms killed 100 per cent of the white mice infected.

RESULTS

Two lots of nutrient broth were employed. The pH of one was adjusted at 6.4 and the pH of the other at 7.2. Though the optimum pH for the growth of *P. pestis*, as will be described in a subsequent paper, is 7.2 to 7.6, for reason to be explained elsewhere we grow cultures for making Haffkine plague vaccine in broth with pH 6.4. Therefore, broths with both these hydrogen ion concentrations were used in these experiments. For experiments with the broth of pH 6.4 the temperatures of incubation employed were 23°C., 25°C., 27°C., 28°C., 29°C., 30°C. and 31°C., and the strain used was 145/Bit. Four to six sets of determinations were made and 8 parallel plates were used for counting the number of organisms in each test tube, giving 32 to 48 counts for each temperature. The results of these counts are given in table 1. For experiments with the broth of pH 7.2, the temperatures of incubation employed were 25°C., 27°C., 28°C., 29°C., 30°C. and 32°C., and the strain used was 55/H. Four sets of experiments were made, and again 8 parallel plates were used for counting the number of organisms in each test tube, giving 32 counts for each temperature. The mean numbers of colonies per plate are given in table 3.

To determine the limiting temperatures of the growth of the organism incubation temperatures of -2°C., 0°C., 2°C., 4°C., 43°C. and 45°C. were

TABLE 1

Colony counts of 36-hour growths of Pasteurella pestis in broth, pH 6.4, in 10 ml. quantities placed in test tubes of 1.7 cm. internal diameter

EXPERIMENT NO.	NUMBER OF COLONIES PER BLOOD AGAR PLATE, 40 SQ. CM., SEEDED WITH 0.05 ML. OF 10^{-4} DILUTION OF THE GROWTHS AT DIFFERENT TEMPERATURES. 8 PARALLEL PLATES USED FOR COUNTING COLONIES FROM EACH GROWTH						
	23°C.	25°C.	27°C.	28°C.	29°C.	30°C.	31°C.
1	8	4	8	8	14	9	11
	6	8	6	15	14	17	10
	6	14	15	17	14	13	4
	9	9	18	20	15	24	10
	7	11	11	11	25	9	6
	9	9	13	8	14	5	9
	6	6	16	8	12	10	8
	10	6	14	11	18	8	4
2	8	18	13	9	18	7	12
	6	20	6	7	21	7	11
	4	13	18	12	17	6	5
	16	6	18	15	17	8	2
	11	7	13	13	10	6	7
	12	9	11	20	12		9
	6	6	16	14	16	13	14
	5		18	12	14	6	6
3	12	9	11	11	14	10	
	8	13	12	12	8	12	
	8	7	17	21	11	7	
	12	7	10	20		8	
	12	15	9	10		11	
	11	17	10	11		5	
	7	7	11	16	15	10	
	8	14	11	13	11	17	
4	14	5	13	10	14	16	10
	4	6	9	22	12	12	9
	12	11	15	7	9	11	11
	12	5	15	14	11	13	9
	15	13	12	16	16	15	9
	12	19	15	20	12	18	9
	10	19	10	15	14	12	8
	10	10	8	9		12	9
5		12	9	6	15	11	9
		14	11	10	13	4	9
		12	7	9	8	5	8
		12	13	10	12	10	16
		4	10	9	17	12	13
		11	8	12	10	15	7
		7	10	8		16	6
		9	9	7		6	10

TABLE 1—Continued

EXPERIMENT NO.	NUMBER OF COLONIES PER BLOOD AGAR PLATE, 40 SQ. CM., SEEDING WITH 0.05 ML. OF 10^{-4} DILUTION OF THE GROWTHS AT DIFFERENT TEMPERATURES. 8 PARALLEL PLATES USED FOR COUNTING COLONIES FROM EACH GROWTH						
	23°C.	25°C.	27°C.	28°C.	29°C.	30°C.	31°C.
6		5 5 3 13 8 12 5 7	11 6 12 16 12 10 15 12		5 14 11 8 19 15 3	9 8 7 6 9 11 6 9	15 10 6 8 10 9 10 6
Mean number of colonies per plate (\bar{x})	9.2500	9.8298	11.9375	12.4500	13.3658	10.2340	8.8500
Mean number of organisms per ml. (millions)	185	197	239	249	267	204	177
Sum of squares of individual colony counts ($\sum x^2$)	3044	5436	7359	6954	7998	5715	3458
Square of the mean \bar{x} number of readings ($n\bar{x}^2$)	2738.00	4541.37	6840.19	6200.10	7324.43	4922.53	3132.90
Sum of squares of deviations from the mean ($\sum (x - \bar{x})^2$)	306.00	894.63	518.81	753.90	673.57	792.47	325.10

TABLE 2

Analysis of data in table 1 to determine whether the observed differences in the growth at different temperatures are statistically significant

TEMPERATURES BETWEEN WHICH THE SIGNIFICANCE OF THE DIFFERENCES IN THE GROWTH IS TESTED	DIFFERENCES BETWEEN THE MEANS $\bar{x}_1 - \bar{x}_2$	SUM OF THE SQUARED DEVIATIONS FROM MEANS AT THE TWO TEMPERATURES $\sum (x - \bar{x})^2 + \sum (x' - \bar{x}')^2$	s	t	REMARKS
°C.					
29-30	3.1318	1466.04	4.1287	3.5495	Highly significant
29-28	0.9158	1427.47	4.2507	0.9694	Not significant
29-27	1.4283	1192.38	3.7020	1.8142	Not significant
27-25	2.1077	1413.44	3.8984	2.6347	Highly significant

employed. Broth with pH 7.2 was used for the determinations. The results are given in table 5. Table 5 also includes the mean values of growths at 25°C. to 37°C. based on counts for table 3.

TABLE 3

Colony counts per plate of 36-hour growths of *Pasteurella pestis* cultivated in broth, pH 7.2, in 10 ml. quantities placed in test tubes of 1.7 cm. internal diameter, according to temperature

TEMPERATURE	MEAN NUMBER OF COLONIES PER PLATE*
°C.	
25	13.8064
27	26.0323
28	24.8750
29	23.0937
30	23.1875
32	20.2812

* 4 sets of determinations were made for each temperature; 8 parallel plates were used for counting the organisms in each test tube, making 32 counts for each temperature.

TABLE 4

Analysis of data summarized in table 3 to determine whether the observed differences in the growth at different temperatures are statistically significant

TEMPERATURES BETWEEN WHICH THE SIGNIFICANCE OF THE DIFFERENCES OF GROWTH IS TESTED	DIFFERENCES BETWEEN THE MEANS $\bar{x}_1 - \bar{x}_2$	SUM OF THE SQUARED DEVIATIONS FROM THE MEANS AT THE TWO TEMPERATURES $S(x - \bar{x})^2 + S(x' - \bar{x}')^2$	s	t	REMARKS
°C.					
27-29	2.9386	1833.70	5.4827	2.1267	Significant
27-28	1.1573	1469.52	5.2656	0.8083	Not significant
27-25	12.2259	1653.78	5.2500	9.1681	Highly significant

TABLE 5

Growth in *Pasteurella pestis* in broth, pH 7.2, placed in 10 ml. quantities in test tubes of 1.7 cm. internal diameter

TEMPERATURES OF INCUBATION	INOCULUM PER ML. OF BROTH	NUMBER OF ORGANISMS PER ML. AFTER 36 HOURS' GROWTH	NUMBER OF ORGANISMS PER ML. AFTER 48 HOURS' GROWTH	NUMBER OF ORGANISMS PER ML. AFTER 96 HOURS' GROWTH	NUMBER OF ORGANISMS PER ML. AFTER 192 HOURS' GROWTH
°C.					
-2*	375,000		430,000	400,000	350,000
0	497,500		522,500	675,000	970,000
2	497,500		608,000	943,000	3,367,000
4	530,000		1,155,000		
25	544,000	276,000,000			
27	544,000	520,000,000			
28	544,000	497,000,000			
29	544,000	462,000,000			
30	544,000	464,000,000			
32	544,000	406,000,000			
37	544,000	98,050,000			
43	560,000		56,350,000	26,850,000	7,400,000
45	587,000		nil		

* For making colony counts of the growths incubated at -2°C., the cultures were quickly warmed by placing them in water bath at 37°C. It was found that if the cultures were allowed to attain room temperature (25°C.) slowly by mere exposure to room air, some of the organisms died and lower counts were obtained than were expected. For this reason it is possible that the counts we have given for growths at 0°, 2°, and 4°C. are lower than they should be, because we did not quickly warm our cultures.

DISCUSSION

The suitability of the technique and the medium (blood agar) employed for making counts was checked by the χ^2 test suggested by Fisher (1936). Detailed statistical analyses will be given in a subsequent paper. It would suffice for the present to state that the observed distribution of χ^2 values closely agrees with the expected distribution of χ^2 values for true samples of a Poisson series. Thus the counts, given in table 1 and averaged in table 3, show a high degree of accuracy.

To verify whether the apparent differences between the mean counts at different temperatures given in tables 1 and 3 are statistically significant, the method of analysis of variance was employed. Values of z for the two sets of experiments given in table 1 and summarized in table 3 are 1.045 and 1.450, respectively. These values indicate that the variations in the counts at different temperatures are due to the significant effect of temperature and not to random sampling errors. Having satisfied ourselves on this point, it only remained to determine the range of optimum temperature of growth. For this purpose the t test (Fisher, 1936) was employed, and the results of analysis are given in tables 2 and 4. The statistical analysis shows that in the broth with optimum hydrogen ion concentration for the growth of *P. pestis*, pH 7.2, maximal growth took place between 27°C. and 28°C. It is to be noted that the growth at this temperature was about five times the growth at 37°C. In the broth with a comparatively unfavourable hydrogen ion concentration, pH 6.4, the growth was less and the zone of optimum growth was slightly widened out to 27°C. to 29°C.

For determining the limiting temperatures of growth the cultures were incubated at -2°C., 0°C., 2°C., 4°C., 43°C. and 45°C. Since the growth at these temperatures was likely to be slow or none at all, cultures were incubated longer than the 36-hour period employed for the optimum growth determinations, for periods varying from 48 hours to 192 hours. If the observations had been limited to 36-hour periods of growth, 0°C. would have appeared as the lower limiting temperature, since no growth resulted at this temperature in even 48 hours.

CONCLUSION

In nutrient broth the optimum growth temperature for *Pasteurella pestis* was found to be 27°C. to 28°C. The growth at this temperature was about five times the growth at 37°C. The limiting growth temperatures were -2°C. and 45°C.

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OPTIMUM AND LIMITING HYDROGEN ION CONCENTRATIONS FOR THE GROWTH OF THE PLAGUE BACILLUS IN BROTH

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Early workers had found that *Pasteurella pestis* grew best in a medium neutral to litmus and that even slightly acid reaction retarded growth (Albrecht and Gohn, 1900). Since the introduction of more accurate methods of measuring the reaction of culture media, only two authors have reported on the subject and both found the plague bacillus to grow best in slightly acid media. Dernby (1921) found the optimum range for the growth of *P. pestis* to be pH 6.5 to pH 7.1 and the limits of growth to be pH 5.6 and pH 7.5. He used unbuffered veal broth as the medium and employed a colorimetric method for adjusting the hydrogen ion concentration of his medium. The growth was estimated by the turbidity method. He gives no description of the strain of organisms used. D'Aunoy (1923) worked with veal infusion broth and buffered it with Na_2HPO_4 and KH_2PO_4 . He buffered his medium to keep the hydrogen ion concentration constant throughout the period of incubation of the cultures. He used both fresh and stock strains of the organism. With freshly isolated strains he found maximum growth to occur between pH 6.0 and pH 6.6, and the limiting pH values to be pH 5.4 and pH 7.6. With stock cultures the maximum growth occurred between pH 6.2 and pH 7.0, and the limiting values were pH 5.0 and pH 8.2.

Paucity of data and contradictory findings led us to reinvestigate the subject. The results are given in this paper. We used the same broth as was employed for determining the optimum temperature of growth of the organism (Sokhey and Habbu 1943). The two strains of the organism used were also the same, 145/B and 55/H. Both were highly virulent first subcultures. The experimental technique was the same.

In some of our early experiments we used broth buffered with sodium and potassium phosphates and found that the addition of these salts in as small a quantity as 0.928 gm. percent concentrations markedly reduced the growth during a 36-hour period of incubation. Buffering also had the effect of flattening out the growth curve, thus giving an apparently wide zone of optimum growth. In buffered broth the maximal growth after 36-hour incubation at 28°C. was on an average 235 million as against 550 million organisms per ml. in unbuffered broth. We have therefore used unbuffered broth in the experiments reported in this paper. This was no handicap as we found that even in unbuffered broth pH did not change during a 36-hour period of incubation. The hydrogen ion concentration of our medium was adjusted electrometrically with the use of normal HCl and NaOH solutions.

TABLE 1

Colony counts of 36-hour growths of *Pasteurella pestis* in broth placed in 10 ml. quantities in test tubes of 1.7 cm. internal diameter

EXPERIMENT NO.	NUMBER OF COLONIES PER BLOOD AGAR PLATE, 40 SQ. CM., SEEDED WITH 0.05 ML. OF 10^{-4} DILUTION OF THE GROWTHS AT DIFFERENT pH VALUES 8 PARALLEL PLATES USED FOR COUNTING COLONIES FROM EACH GROWTH								
	6.2	6.6	6.8	7.0	7.2	7.4	7.6	7.8	8.0
1	10	29	28	22	22	26	22	25	19
	11	19	26	29	30	23	35	21	17
	18	20	13	33	22	18	27	25	12
	13	23	16	30	37	31	34	25	13
	5	16	18	30	36	21	29	17	16
	11	20	23	29	34	18	40	21	26
	14	26	20	24	23	26	26	16	17
	18	17	18	24	41	37	26	14	18
2	16	23	23	31	20	31	20	26	16
	13	18	21	19	23	22	22	22	18
	17	21	22	23	35	35	32	26	9
	20	13	18		30	32	29	17	23
	11	17	15	25	27	27	33	24	18
	14	16	24	20	20	22	18	18	16
	13	24	17	29	23	28	34	10	19
	18	26	20	25	39	23	28	24	26
3	13	14	11	21	35	22	19	29	18
	18	26	17	29	28	18	31	22	21
	10	22	17	32	36	36	18	31	20
	14	14	10	18	33	26	22	15	25
	14	18	21	27	33	32	21	29	16
	8	18	21	15	31	20	25	24	22
	10	17	21	22	41	41	27	21	17
	11	13	16	17	31	25	35	33	12
4	13	16	19	13	20	45	25		12
	14	21	16	13	23	30	23		22
	20	26	20	21	26	35	17		28
	12	17	16	24	25	32	25		13
	14	23	16	14	19	34	14		24
	22	16	16	21	26	40	19		11
	10	25	14	18	27	33	23		13
	16	21	15	17	33	24	33		16
5	5	18	22	17	18		35		18
	16	10	23	26	23		16		25
	18	20	16	25	37		17		22
	20	15	24	38	20		31		25
	11	20	25	24	24		27		12
	13	16	17	24	43		35		22
	15	19	26	27	27		18		19
	10	20	31	23	22		20		19
Mean number of colonies per plate (\bar{x})	13.7250	19.3250	19.3000	23.5641	28.5750	28.5312	25.8250	22.2916	18.3750
Mean number of organisms per ml. (millions)	274	386	386	471	571	571	516	446	367
Sum of squares of individual colony counts (Σx^2)	8139	15649	15710	22955	34563	27625	28359	12657	14389
Square of the mean Σ number of readings ($n\bar{x}^2$)	7535.02	14938.22	14899.60	21655.22	32661.22	26048.94	26677.22	11925.97	13505.62
Sum of squares of deviations from the mean ($\Sigma (x-\bar{x})^2$)	603.98	710.78	810.40	1299.78	1901.78	1576.06	1681.78	731.03	83.888

RESULTS

Two strains of the organisms were used and the results of the first series of experiments are given in table 1 and the results of the second series are summar-

TABLE 2

Analysis of data in table 1 to determine whether the observed differences in growth at different pH values are statistically significant

pH VALUES BETWEEN WHICH THE SIGNIFICANCE OF THE DIFFERENCES IN THE GROWTH IS TESTED	DIFFERENCES BETWEEN THE MEANS $\bar{x}_1 - \bar{x}_2$	SUM OF THE SQUARED DEVIATIONS FROM THE MEANS AT THE TWO pH VALUES $S(x - \bar{x})^2 + S(x' - \bar{x}')^2$	s	t	REMARKS
7.2-7.0	5.0109	3201.56	6.4481	3.4532	Highly significant
7.2-7.4	0.0438	3477.84	7.0486	0.0262	Not significant
7.2-7.6	2.7500	3583.56	6.7781	1.8143	Not significant
7.2-7.8	6.2834	2632.81	6.5164	3.7345	Highly significant

TABLE 3

Colony counts per plate of 36-hour growths at different pH values of Pasteurella pestis cultivated in broth seeded with .05 ml. of 10^{-6} dilution

pH VALUE	MEAN NUMBER OF COLONIES PER PLATE*
6.2	8.6250
6.6	18.6383
6.8	21.3541
7.0	21.3541
7.2	23.6808
7.4	25.7083
7.6	23.2708
7.8	19.2500
8.0	16.2500

* 6 sets of determinations were made for each pH value; 8 parallel plates were used for counting the organisms in each test tube, making 48 counts for each value.

TABLE 4

Analysis of data summarized in table 3 to determine whether the observed differences in growth at different pH values are statistically significant

pH VALUES BETWEEN WHICH THE SIGNIFICANCE OF THE DIFFERENCES IN THE GROWTH IS TESTED	DIFFERENCES BETWEEN THE MEANS $\bar{x}_1 - \bar{x}_2$	SUM OF THE SQUARED DEVIATIONS FROM THE MEANS AT THE TWO pH VALUES $S(x - \bar{x})^2 + S(x' - \bar{x}')^2$	s	t	REMARKS
7.4-7.0	4.3542	3139.12	5.7788	3.6912	Highly significant
7.4-7.2	2.0275	2688.33	5.3765	1.8376	Not significant
7.4-7.6	2.4375	3431.57	6.0420	1.9763	Just significant

ized in table 3. In these experiments the range of pH values was from 6.2 to 8.0. For determining limiting pH values, separate tests were made with pH values extending as low as 4.8 and as high as 9.8. Because with these pH values

TABLE 5

Growth of Pasteurella pestis in broth of different pH values placed in 10 ml. quantities in test tubes of 1.7 cm. internal diameter

pH OF THE MEDIUM (BROTH)	INOCULUM PER ML. OF THE MEDIUM (BROTH)	NUMBER OF ORGANISMS PER ML. AFTER 36 HOURS' GROWTH	NUMBER OF ORGANISMS PER ML. AFTER 48 HOURS' GROWTH
4.8	380,000		Nil
5.0	445,000		1,500
5.4	445,000		45,800,000
5.8	680,000	49,500,000	
6.2	680,000	223,000,000	
6.6	680,000	379,000,000	
6.8	680,000	406,000,000	
7.0	680,000	449,000,000	
7.2	680,000	522,000,000	
7.4	680,000	542,000,000	
7.6	680,000	490,000,000	
7.8	680,000	415,000,000	
8.0	680,000	346,000,000	
9.0	380,000		157,000,000
9.2	445,000		157,000,000
9.4	445,000		96,400,000
9.6	415,000		580
9.8	415,000		Nil

TABLE 6

Analysis of data showing the close correlation between the observed and the expected χ^2 values obtained from 143 sets of 8 parallel plates reported in table 1 and summarized in table 3 of this and the previous paper (Sokhey and Habbu 1943)

	OBSERVED FREQUENCIES (a)	EXPECTED FREQUENCIES (b)	$\frac{(a-b)^2}{b}$
0	6	7.15	0.184
2.167	6	7.15	0.184
2.833	14	14.30	0.006
3.822	11	14.30	0.761
4.671	23	28.60	1.096
6.346	30	28.60	0.068
8.383	15	14.30	0.034
9.803	18	14.30	0.957
12.017	10	7.15	1.135
14.067	10	7.15	1.135
Total	143	143	5.560*

* P for this value lies close to 0.7.

poorer growth was expected, the period of incubation was increased to 48 hours. The results are given in table 5. Table 5 includes also the mean values of growths between pH 6.2 and pH 8.0 based on counts for table 3.

DISCUSSION

The basis of the work reported in this paper and the previous one (Sokhey and Habbu 1943) is the technique of colony count employed. It is therefore important that the accuracy of the method be assessed. The colony counts reported in table 1 and summarized in table 3 of this and the previous paper are based on 143 sets of 8 parallel plates each, exclusive of the sets of counts in which some plates had been contaminated. These data are sufficiently large to permit of a statistical evaluation by the χ^2 test suggested by Fisher (1936) for the purpose. The results of such an analysis are given in table 6. The observed distribution of χ^2 values closely agrees with the expected distribution of χ^2 values for true samples of Poisson series; the value of P lies very close to 0.7. Thus the technique of colony count employed by us undoubtedly achieves a high degree of accuracy.

As in the previous paper on the optimum temperature of growth, the data were statistically analysed by the method of analysis of variance, and values of 1.6920 and 1.9034, respectively, were obtained for z for the two series. The range of optimum pH for the growth of the plague bacillus was determined by employing the t test. The results are given in tables 2 and 4. This statistical analysis shows that optimum growth results between pH 7.2 and pH 7.6. The data reported in table 5 show that the limiting pH values are pH 5.0 and pH 9.6. It is to be noted that though maximum growth occurs between pH 7.2 and 7.6, good growth occurs over a wide zone, pH 6.6 to pH 8.0.

CONCLUSION

In nutrient broth, at the optimum temperature of growth (28°C.) the limiting pH values for the growth of *Pasteurella pestis* were found to be 5.0 and 9.6 and though maximum growth occurred between pH 7.2 to 7.6, fairly good growth was obtained over a wide zone, pH 6.6 to pH 8.0.

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THE EFFECT OF SOLID SURFACES UPON BACTERIAL ACTIVITY¹

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The bacterial population of water samples from the sea, lakes or other sources usually increases during storage. While several different factors may be operative (Prescott and Winslow, 1931), it is noteworthy that the magnitude of the increase is often related to the size of the receptacle in which the water is stored. For example, Whipple (1901) found that the bacterial population of water, which initially contained an average of 77 bacteria per ml., increased to 300 per ml. in a gallon, 900 per ml. in a quart, 7,020 per ml. in a pint and 41,400 per ml. in 2-ounce bottles after 24 hours' incubation under comparable conditions. He attributed this to the greater availability of oxygen in the small receptacles which were not filled to capacity. However, ZoBell and Stadler (1940) have shown that the multiplication and respiration of aerobic bacteria is independent of the oxygen tension within the examined ranges of 0.30 to 36 mgm./liter.

Using oxygen consumption as well as bacterial multiplication in glass-stoppered bottles filled to capacity with sea water as criteria, ZoBell and Anderson (1936) noted that bacteria are generally more active in small than in large receptacles of similar shape. Since the small receptacles present relatively more solid surface per unit volume of stored water than large receptacles, they concluded that solid surfaces are beneficial to bacteria in dilute nutrient solutions. A similar conclusion was reached by Lloyd (1937). The following report is concerned with the ways in which solid or adsorbing surfaces may influence bacterial activity.

GLASS SURFACES ADSORB NUTRIENTS

Inasmuch as the effect of volume or solid surfaces upon bacterial activity can be demonstrated only in very dilute nutrient solutions and since the effect is more pronounced with colloidal than with dissolved nutrients, ZoBell (1937) suggested that nutrients may be concentrated on solid surfaces. This explanation is supported by more recent observations here and elsewhere. The work of ZoBell and Grant (1943) shows that bacterial activity is directly proportional to the concentration of nutrients when the latter is less than 10 mgm./l. Since sea water ordinarily contains less than 5 mgm./l. of organic matter (Krogh, 1931), only a small part of which is readily attacked by bacteria (Waksman and Carey, 1935a), it follows that any factor which tends to concentrate the organic matter would promote bacterial activity.

Although the small quantity and complexity of the organic content of sea water make it difficult to estimate the amount which is adsorbed by glass or other solid surfaces under different conditions, there are several ways in which

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it can be demonstrated that organic material is adsorbed. The sea water used for this purpose was collected in carboys, passed through a fine sintered-glass filter and stored in a water bath at 0°C. The low temperature inhibits (but does not prevent) bacterial activity while the organic matter is being adsorbed.

The glassware used in the experiments was thoroughly cleaned in hot sulfuric-acid-dichromate-cleaning-solution, treated with trisodium phosphate solution and finally thoroughly rinsed with distilled water. Glass beads and glass wool were heated in a muffle furnace for three hours to free them of organic matter. There is no evidence that the glass carried any nutrients or reactive material. Pyrex glass was used almost exclusively, although no differences were found in the amount of organic matter adsorbed, or in the attachment of bacteria, on Pyrex glass and other kinds of glass.

After standing in sea water at 0°C. for several days, glass slides were removed and stained with methylene blue, gentian violet and carbol fuchsin. Microscopic examination showed the presence of numerous bacteria covering one-fiftieth to one-tenth of the surface. All of the surface was coated with an irregular film of material which stained like complex organic matter. There was little or none of this stained film on slides which were immersed in sea water for only a short period of time. Likewise there was no film on slides immersed in "aged" sea water, the organic content of which had been reduced to less than 0.5 mgm./l.

In other experiments sea water was stored at near 0°C. in 600 ml. bottles partly filled with glass wool, glass beads or with glass tubes to give 2 to 200 cm.² of glass surface per ml. of solution. After different periods of storage the water was carefully siphoned out of the bottles and 100 ml. of dilute permanganate solution was introduced. While heating the bottles in a boiling water bath, they were manipulated to rinse the interior with the oxidizing agent after which they were cooled and the contents titrated with N/80 thiosulfate. The procedure indicated that from 2 to 27 per cent of the organic content of the sea water had been adsorbed by the glass. The amount adsorbed was roughly proportional to the area of solid surface exposed to the water. Repeating this experiment with dilute aqueous solutions or suspensions of known chemical composition showed that glucose, lactate and glycerol were not adsorbed perceptibly, while lignoprotein, nutrose and an emulsified chitin preparation were adsorbed. Very little peptone was adsorbed.

Using similar methods Stark *et al.* (1938) found that measurable amounts of organic matter accumulate within a few hours on chemically clean glass slides immersed in lake water. They expressed the belief that the accumulation of organic nutrients favors bacterial growth. Corroborative evidence is given by the studies of Heukelekian and Heller (1940) on the relation between food concentration and solid surfaces.

Harvey (1941) noted that small glass tubes immersed in sea water adsorbed enough organic matter to alter the surface tension as shown by capillary attraction. Treating a solution containing 10 mgm./l. of peptone with permanganate before and after being exposed to enough glass wool to give a surface of 50 cm.²/

ml. of solution, he found that from 0 to 7 per cent of the peptone was adsorbed by the glass wool. Although the amount of organic matter adsorbed is barely more than the range of the experimental error, Harvey speculates that there might be enough to give a monomolecular layer of nutrient on the glass which might be replaced as fast as it is utilized by bacteria. He points out that according to Blodgett (1935), layers many molecules thick may build up on solid surfaces. A key to the voluminous literature on the factors influencing the adsorption by solids is given by Adam (1938).

BIOLOGICAL EVIDENCE OF ADSORPTION

Immediately after its collection and filtration through fine sintered glass filters, sea water was dispensed in 145 ml. glass-stoppered bottles. Part of the bottles were loaded with several pieces of thin-walled glass tubing tightly packed in an upright position, which materially increased the area of solid surface ex-

TABLE 1

Oxygen consumed by bacteria after different periods of time in sea water stored at near 0°C. and at 22°C. in glass-stoppered bottles, some of which were filled with glass tubes to increase the area of solid surface

	GLASS-STOPPERED BOTTLES		BOTTLES WITH GLASS TUBES	
	Ratio of cm. ² :ml.			
	11:1		7.2:1	
	Incubation temperature (°C)			
	22		22	
Oxygen consumed in 5 days (mgm./l.)	0.02	0.37	0.01	0.46
Oxygen consumed in 10 days (mgm./l.)	0.12	0.78	0.20	0.91
Oxygen consumed in 20 days (mgm./l.)	0.26	1.02	0.38	1.29

posed to water without appreciably decreasing the volume. The oxygen content of the water in some of the bottles was determined at once by a refined Winkler method which was accurate to ± 0.01 mgm./l. Part of the bottles were stored in a water bath at 22°C. and part of them were stored in a water bath at near 0°C. Bottles were removed for analysis after 5, 10 and 20 days' incubation. The protocol of a representative experiment is summarized in table 1.

As might be expected, bacteria multiplied more rapidly and consumed more oxygen in the water incubated at the higher temperature. At both temperatures more oxygen was consumed in the water which was exposed to the largest solid surface. Previous work has shown that the amount of oxygen consumed is directly proportional to the amount of organic matter oxidized. It requires approximately 1 mgm. of oxygen to oxidize 1 mgm. of organic matter found in sea water. For each milligram of organic carbon which is oxidized to carbon dioxide, 0.45 to 0.65 mgm. of organic carbon is converted into bacterial protoplasm (Waksman and Carey, 1935b, ZoBell and Grant, 1943).

The plate count of the water stored at 0°C. increased progressively throughout the experiment. The plate count of the water stored at 22°C. reached a maximum of several million bacteria per ml. in 5 days and then dropped off sharply after 10 days. Although more oxygen was consumed at 22°C. in the bottles which presented the larger surface area from the 5th to the 20th day, the plate count of this water was smaller than in the bottles having no glass tubes to increase the solid surface. This apparent paradox is explained by the abundance of periphytic or sessile bacteria found attached to the glass. From the direct microscopic examination of the water itself and glass slides immersed in the water, it was calculated that there were more bacteria tenaciously attached to the walls of small glass bottles than the number found in the water.

In earlier experiments ZoBell and Anderson (1936) found 31,000 bacteria per ml. of water and an equivalent of 84,000 bacteria per ml. attached to the glass. Sometimes ten times as many bacteria are found attached to the glass as in the

TABLE 2

Oxygen consumed by bacteria in 20 days at 22°C. in sea water, which had been stored in 145 ml. glass-stoppered bottles for 5 days at 0°C.

Part of the water was left in the original bottles in which it was stored and part was transferred to clean bottles and the emptied "original" bottles filled with aged sea water. Aged sea water was incubated in clean bottles as a control.

	INITIAL DISSOLVED OXYGEN CONTENT	DISSOLVED OXYGEN AFTER 20 DAYS	OXYGEN CONSUMED IN 20 DAYS
	mgm./l.	mgm./l.	mgm./l.
Original water in original bottles... ..	7.83	6.93	0.90
Original water in clean bottles.....	7.86	7.18	0.68
Aged water in original bottles.....	7.62	7.23	0.39
Aged water in clean bottles	7.61	7.47	0.14

water. The ratio of the number of bacteria occurring in the water and on solid surfaces seemed to be influenced by the concentration and kind of organic matter present, the proximity of the solid surface to the water mass, the time and temperature of incubation and the kinds of bacteria present.

The water in some of the bottles was carefully siphoned into sterile chemically clean bottles after 5 days storage at 0°C. The original bottles were drained free of sea water and refilled with aged sea water which contained less than 0.2 mgm./l. of bacteriologically oxidizable organic matter. The decanted water with which the bottles were filled originally and the aged sea water in the original bottles was then incubated at 22°C. for 20 days. Oxygen determinations revealed that detectable quantities of oxidizable organic matter had been adsorbed by the glass of the original bottles as shown by the data in table 2. The data are the average of quadruplicates.

It will be noted that whereas the original water, which was incubated in the bottles (original) in which it had been stored for 5 days at 0°C., consumed an average of 0.90 mgm./l. of oxygen in 20 days at 22°C., water siphoned from the original bottles into chemically clean bottles consumed only 0.68 mgm./l. of

oxygen. The difference is believed to represent the amount of organic matter adsorbed by the glass walls of the original bottle. This interpretation of results is substantiated by the fact that aged sea water consumed 0.25 mgm./l. more oxygen when incubated in the "original" bottles than when incubated in chemically clean bottles.

Figure 1 shows the number of bacteria found by plate counts in sea water stored in glass-stoppered bottles at 12°C. and the number of attached bacteria. The number of attached bacteria per ml. of water was estimated by counting microscopically those attached per unit area of glass slides submerged in the water and multiplying by the total area of glass exposed to the water. After ten days' incubation the water from part of the bottles was siphoned into "new"

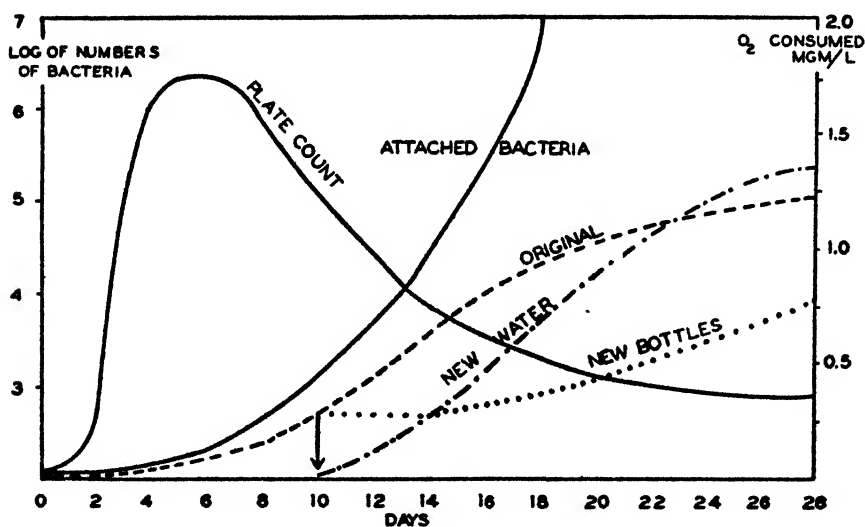


FIG. 1. Number of bacteria found in sea water as indicated by plate counts and the equivalent number per ml. attached to glass surfaces after different periods of incubation at 12°C. (solid lines). The oxygen uptake in the water is also shown (original). After 10 days part of the water was transferred to chemically clean (new) bottles and the emptied bottles were refilled with recently collected (new) water.

chemically clean bottles and the emptied bottles were refilled with "new" recently collected sea water. Determining the oxygen content of water after different periods of incubation showed that little more oxygen was consumed in the decanted or original water in "new" bottles, presumably because a large proportion of its bacterial and organic content had been adsorbed by the original bottle in which it was stored. That both organic matter and bacteria were adsorbed is indicated by the fact that the "new" water in the original bottles consumed more oxygen and consumed it more rapidly than the original water in the original bottles.

The experiment has been repeated several times with similar results. However, in some lots of water the beneficial effect of solid surface upon bacterial activity is barely perceptible, which may be attributable to the quality and

quantity of organic matter, the kinds of bacteria present, the surface tension of the water, the nature of the electrolytes and other factors which are being investigated. The explanation of the negative or indifferent results like those obtained by Castell and McDermott (1942), for example, require further experimental work.

The amount of adsorbable organic matter present in natural waters might be influenced by the bacterial population of the water as well as by the quality of the organic matter itself. Part of the organic matter is actually in the form of attached bacterial cells. During the early stages of storage amorphous organic matter appears to predominate on submerged surfaces. After prolonged storage, glass surfaces are usually completely covered with attached bacteria which obliterate any amorphous organic matter which might remain. The work of ZoBell and Grant (1943) indicates that, after prolonged incubation, 60 to 70 per cent of the oxidizable organic carbon in dilute solutions is oxidized to carbon dioxide, and 30 to 40 per cent is converted into bacterial protoplasm.

Our work (ZoBell, *et al.*, 1943) on bacteria which oxidize petroleum hydrocarbons has supplied the most outstanding examples of the beneficial effect of adsorbing surfaces. Due to the low solubility and relative immiscibility in water, hydrocarbons are attacked very slowly by bacteria in aqueous media. If, however, the hydrocarbons are made available on the extensive surfaces of adsorbents such as sand, shredded asbestos or infusorial earth, the rate of bacterial utilization is accelerated two- to ten-fold. Söhngen (1913) noted that ignited soil, silica or iron oxide promoted the bacterial oxidation of petroleum. He concluded that the growth of the bacteria was a function of the solid surface. Similarly Greig-Smith (1914) found that kieselguhr accelerated the utilization of paraffin hydrocarbons by bacteria.

SOME BACTERIA ARE SESSILE

Many species of bacteria grow tenaciously attached to solid surfaces. This is the basis of the buried slide technique of Cholodny (1930) for the direct microscopic study of soil bacteria. It is also the basis of the submerged slide technique of Henrici (1933) for an ecologic survey of water bacteria. The work of ZoBell and Allen (1935), Henrici (1936), Hotchkiss and Waksman (1936), Smith and ZoBell (1937), and Waksman and Vartiovaara (1938) indicates that many water bacteria grow attached to solid surfaces. Kusnetzowa (1937) believes that all water bacteria are capable of attaching themselves to glass. Henrici and Johnson (1935) have described several species of periphytic bacteria which grow only attached to a firm substrate.

Exclusive of exotic species from the terrestrial environment along the littoral zone, most of the bacteria found in the sea appear to be associated with solid surfaces. The microscopic examination of suspended particles reveals the presence of numerous bacteria, whereas very few bacteria are found unattached in sea water. This is in agreement with the observation of Lloyd (1930) that marine bacteria are not planktonic but are attached to solid particles. According to Waksman *et al.* (1933) the bacteria of the sea are usually found attached

to larger plankton organisms, existing "only to a very limited extent in the free water of the sea."

The most obvious reason bacteria are found associated with plankton organisms is because the tissue and organic metabolic products of the latter provide a ready source of bacterial food and energy. Also plankton organisms are often coated with a slimy exudate to which bacteria adhere. However, many bacteria do not depend for attachment upon the adhesive properties of solid surfaces, and they are not entirely organotropic because they attach to chemically clean glass, porcelain, plastic, metal and other surfaces. Bacteria will be found tenaciously attached to glass slides an hour or two after submerging the slides in the sea. Such bacteria seem to be stereotropic or thigmotactic.

Upon isolation from submerged slides some of the bacteria grow only on solid surfaces. We have observed sessile bacteria which form a film on the walls of test tubes or flasks without perceptibly clouding dilute nutrient solutions. The most striking example is a chitinoclastic bacterium (ZoBell and Rittenberg, 1938) which grows on strips of chitin with virtually no bacteria occurring in the liquid medium as indicated by the fact that no bacteria may be found in a 2-mm. loopful of fluid although masses of them can be observed growing on solid surfaces.

Achromobacter marinoglutinosus, *Achromobacter membranoformis* and *Flavobacterium amocontactum* are new species of sessile, film-forming or attachment bacteria described by ZoBell and Allen (1935). Dr. Robert S. Breed directs my attention to the fact that since the first two organisms have lophotrichous or polar flagella, according to the revised key they should be designated *Pseudomonas marinoglutinosus* and *Pseudomonas membranoformis*. Among the new species of marine bacteria recently described by ZoBell and Upham (1943) are several sessile forms including *Pseudomonas stereotropis*, *P. sessilis*, *P. periphyta*, *P. coenobios*, *P. membranula*, *Bacillus epiphyticus*, *Micrococcus sedentarius*, *M. sedimenteus*, *Achromobacter stationis*, *A. aquamarinus*, *Bacterium sociiovivum* and *B. immotum*.

A total of 96 different cultures of bacteria isolated from marine materials have been used to inoculate sea water in wide mouth bottles enriched with 100 mgm./l. of peptone. Sterile chemically clean glass slides were inserted vertically. After different periods of incubation at 25°C., a slide from each bottle was removed. Without fixation the slides were washed, stained and dried. Large numbers of bacteria were found on slides from 29 of the cultures, virtually no bacteria were found on slides from 47 cultures and a variable number of bacteria were found on slides from 20 cultures. Although the procedure does not differentiate clearly between sessile bacteria and non-sessile ones, it shows differences in the attachment propensities of certain bacterial species which can be duplicated.

No relationship was found between the gram reaction of the pure cultures and their attachment propensities. This is significant because according to Eisenberg (1918), gram-positive bacteria of high lipid content are adsorbed more readily than gram-negative ones. Rubentschik *et al.* (1936) observed no

sharp dividing line between the adsorptive capacity of gram-negative and gram-positive species, although they did note differences in the sessile habits of various species of bacteria.

The development of micro-colonies on glass slides indicates that some of the sessile bacteria multiply while attached to solid surfaces. In very dilute nutrient solutions more bacteria will often be found growing on the walls of the culture receptacle and on immersed glass slides than in the solution (ZoBell and Anderson, 1936).

HOW BACTERIA ATTACH

Sessile bacteria do not seem to be covered with a mucilaginous substance which causes them to stick to solid surfaces with which they come into contact. This point has been investigated by immersing glass slides in suspensions of bacteria and removing them after different periods of time for microscopic examination. Very few bacteria attach to slides firmly enough to resist being washed off with running water unless the slides have been left in contact with the bacteria in dilute nutrient solutions for at least a few hours. Likewise only a small proportion of the bacteria transferred to glass slides and air dried, as in the preparation of a smear for staining, adhere to the glass without heat or chemical fixation. However, when the glass slides are left in dilute nutrient solutions of bacteria for several hours, many bacteria adhere so tenaciously that they are dislodged neither by washing with water nor by staining procedures.

The foregoing observations suggest that bacteria grow on, or attach themselves to, solid surfaces rather than merely being passively stuck. Credibility is lent to this conclusion by the fact that regardless of the density of the bacterial population, more bacteria attach to glass slides during the early logarithmic phase of growth than during later growth phases.

Henrici and Johnson (1935) have described a group of bacteria "which secrete stalks by which they are attached to a firm substrate." Their search of the older bacteriological literature has shown that similar types have been observed before. We have found bacteria growing on slides submerged in the sea and in Great Salt Lake (Smith and ZoBell, 1937) which have a definite holdfast, some of which could be described as having "stalks."

As pointed out by Henrici and Johnson (1935), it is often difficult to distinguish bacteria having large polar or adherent lophotrichous flagella from stalked bacteria on slides which, after several days submergence, are covered with various kinds of bacteria besides other sessile organisms and a heterogeneous assortment of particulate and amorphous organic matter. Also in some cases we have not been certain that some of the definitely stalked microorganisms appearing on submerged slides were bacteria although, except for the stalk, they had the morphological appearance of bacteria. However, in spite of the difficulties, careful microscopic study of submerged slides has convinced us that some water bacteria are attached to solid surfaces by means of a filament or stalk which is definitely longer and narrower than the bacterial cell itself. In fact, some stalked bacteria have been observed which appeared to be dividing.

On only three slides out of several hundred examined have we observed bac-

teria with stalks which were sufficiently rigid to hold the bacteria away from the slide. When examined as wet mounts the bacteria appeared to be a few microns away from the slide. It was necessary to focus up and down in order to follow the stalk from the holdfast to the bacterial cell.

Several cultures of sessile bacteria have been isolated from submerged slides. After a few hours submergence in the sea the glass slides were washed with sterile water, transferred aseptically to sterile petri dishes and covered with nutrient agar (ZoBell, 1941). When grown in sea water enriched with 5 mgm./l. each of glucose and peptone, several of these cultures contained bacteria which attached to glass slides immersed in the medium. Six of them had structures which appeared to be stalks, but with all six cultures the bacterial cell, the stalk and the holdfast rested directly upon the glass slide. Upon cultivation on solid media they lose their stalks, although this property is regained after a few passages through sea water containing less than 10 mgm./l. of organic matter.

Only a small percentage of the cells of the six "stalked" cultures found attached to glass slides exhibited stalks. This may be attributed to faulty technique in staining stalks; it may suggest that the stalks develop after the cells have attached, or it is possible that all the cells do not have stalks. Further studies will be necessary to clarify this point.

Although some bacteria are attached by stalks, this is not the commonest attachment structure. Most sessile bacteria are found with the bacterial cells in intimate contact with the solid surface. It is believed that after coming into contact with a solid surface, physiologically active sessile bacteria secrete a cementing substance. When the bacteria are removed mechanically from glass slides to which they have attached themselves, a faintly staining film having the shape and arrangement pattern of the attached cells frequently remains on the slides.

Some bacteria appear to secrete a faintly staining material on the slide which is two or three times the diameter of the cells themselves. Examining slides after different periods of submergence in bacterial cultures reveals that the size of this film increases with age. It is not uncommon to see micro-colonies on "islands" of the film. The smaller islands conform more or less to the shape of the cells but larger islands of the film are quite irregular in outline.

The film appears to be a part of or product of the bacterial cell. The fact that the islands are most pronounced in dilute nutrient solutions makes it seem probable that the film does not consist of organic matter adsorbed from the water. However, it is still indeterminate whether the bacteria are responsible for the presence of the film or if the film of adsorbed organic matter precedes the bacteria thereby providing for their attachment. Microscopic particles of detritus including particles of carbon and indigotin, with which the slides have been treated, stick to the film thereby establishing that the film has adhesive properties. This mucilaginous slime produced by sessile bacteria is believed to be instrumental in the fouling of submerged surfaces (ZoBell and Allen, 1935, ZoBell, 1939). According to Sanborn (1932), the formation of slimy or viscous growths is common in cultures from marine fish.

An effort has been made to characterize the holdfasts of sessile bacteria by

treating slides coated with attached bacteria with different solvents. Dilute HCl or H₂SO₄ has little or no effect on the holdfasts, which indicates that the cementing or adhesive material is not calcareous. (Many sessile organisms attach by means of calcareous cements which are dissolved by dilute acids.) Dilute caustic, ammoniacal or bicarbonate solutions were without apparent effect. The holdfast is not destroyed by 95 per cent alcohol. Xylol, chloroform, ether and carbon disulfide seemed to dislodge some of the attached bacteria but not enough to warrant the conclusion that the cement is a lipin. Eisenberg (1918) believed that a high lipin content favored the adsorption of bacteria.

None of the common staining solutions dislodged the attached bacteria. When dessicated, many of the attached bacteria disappear from slides. Recently it has been found that a bacterial chitinase extract dislodges many attached bacteria which suggests that the holdfast is chitinous, but the extract also contains proteolytic and possibly other exoenzymes which themselves might have digested the cementing material.

Dilute solutions of detergents or wetting agents such as sodium taurocholate, sodium ricinoleate, sodium lauryl sulfate (Dreft) or "aerosol" remove part of the attached bacteria, the cells of some cultures being affected more than those of others. The presence of detergents or surface tension depressants in dilute nutrient solutions retards the attachment of bacteria to solid surfaces.

When submerged in the sea the number of bacteria found on glass slides increases exponentially with time until the bacteria became too numerous to count or are obscured by other organisms and detritus which attach simultaneously. This suggests that the bacteria are multiplying on the glass or that the attachment of bacteria promotes the attachment of additional bacteria. Probably the adhesive films formed by the first sessile bacteria help other bacteria which come into contact with the surface to gain lodgement long enough to produce their own holdfasts. If only the cumulation of adsorbed nutrients was responsible, the increase in the number of bacteria on submerged slides would probably be arithmetic rather than exponential.

EFFECT OF ORGANIC NUTRIENTS

Unquestionably organic nutrients on the solid surfaces promote the multiplication of bacteria but there is evidence that adsorbed nutrients are not directly responsible for the attachment of bacteria. Slides have been left in sterile sea water until they have been covered with adsorbed organic matter after which they were submerged in the sea. Significantly more bacteria did not attach to these slides than to chemically clean ones.

However, the organic content of the water has a pronounced effect on the relationship of microorganisms to solid surfaces. Increasing the solid surface area ordinarily does not alter the rate of bacterial multiplication or metabolism in solutions containing more than 5 to 10 mgm./l. of organic nutrients. The beneficial effect of solid surface becomes more pronounced as the concentration of organic nutrients decreases. Heukelekian and Heller (1940) observed that *Escherichia coli* failed to grow when the concentration of organic nutrients was

less than 0.5 to 2.5 mgm./l. except in the proximity of solid surfaces. When the concentration exceeded 25 mgm./l., the activity of *E. coli* was not influenced perceptibly by solid surfaces.

The kind of organic matter is also important. Increasing the ratio of solid surface to volume does not influence the rate of bacterial activity in dilute solutions (1.0 to 10 mgm./l.) of glucose, glycerol or lactate. Many bacteria are found attached to glass slides in such solutions, but in such solutions the bacteria do not seem to depend upon solid surfaces because proportionately more occur suspended in the solution than anchored. Conversely in solutions enriched with 5 mgm./l. of sodium caseinate, lignoprotein or an emulsified chitin preparation inoculated with a few bacteria per ml., a beneficial effect of increased solid surface can be demonstrated. This suggests that there is a relationship between solubility, dispersion or molecule size of the organic nutrient and the surface phenomenon. However, many other factors may be involved and further studies must precede generalizations.

The beneficial effect of solid surface upon the rate of bacterial activity in sea water is much more pronounced with some lots of sea water than with others. Since the inorganic content, gas tension and the pH of sea water have been nearly constant, the differences are attributed to differences in the organic content of the water. A heterogeneous assortment of organic matter occurs in sea water but most of it probably consists of large molecules of colloidal dimensions which are fairly refractory to bacteria decomposition. Differences may be expected in the relative quantities of different kinds of organic matter adsorbed on glass, and especially differences in the orientation of molecules. Their availability might be influenced by the angle of adsorption as well as by the polarity, both of which might influence the vulnerability of hydroxy, carboxy, amino or other groups. According to Adam (1938) most organic molecules form zero angles with glass but certain large molecules are "adsorbed on to glass with the long chains outwards." For example, the contact angle of paraffin wax molecules is 105° , the largest found with solids and water.

It is generally believed that the large molecules or particulate materials are hydrolyzed by exoenzymes before the organic matter can be ingested and assimilated by bacteria. In a dilute solution of such organic hydrolytes, containing only a relatively few bacteria, the bacteria would have to be in close contact with the hydrolyte in order for the exoenzymes to be most effective. Otherwise the bacterial exoenzymes would be dissipated throughout the solution. It is conceivable that the hydrolyzates themselves might have a tendency to diffuse away from the bacterial cell responsible for their formation; so, in dilute nutrient solutions of large molecules of organic matter, the bacteria may have difficulty in digesting and absorbing or ingesting enough nutrient to provide for their organic requirements.

However, if the bacterial cell is on a solid surface juxtaposed with nutrient hydrolytes, the solid surface may retard the diffusion of the exoenzymes and the hydrolyzates away from the cell (see figure 2). Besides the physical attraction which might be exerted by the solid surface, an anchored bacterium would not

be influenced by the bombardment of molecules (with the resultant Brownian movement and diffusion) which would tend to separate the bacterium from its exoenzymes and hydrolyzates. Furthermore, the interstices at the tangent of the bacterial cell and solid surface might serve as concentration foci for exoenzymes and hydrolyzates. As other bacteria attach or develop from cell division, more interstitial or capillary spaces would be formed which would tend to retard further the diffusion of materials away from the cells and to favor the absorption or ingestion of soluble nutrients. Also solid surfaces might

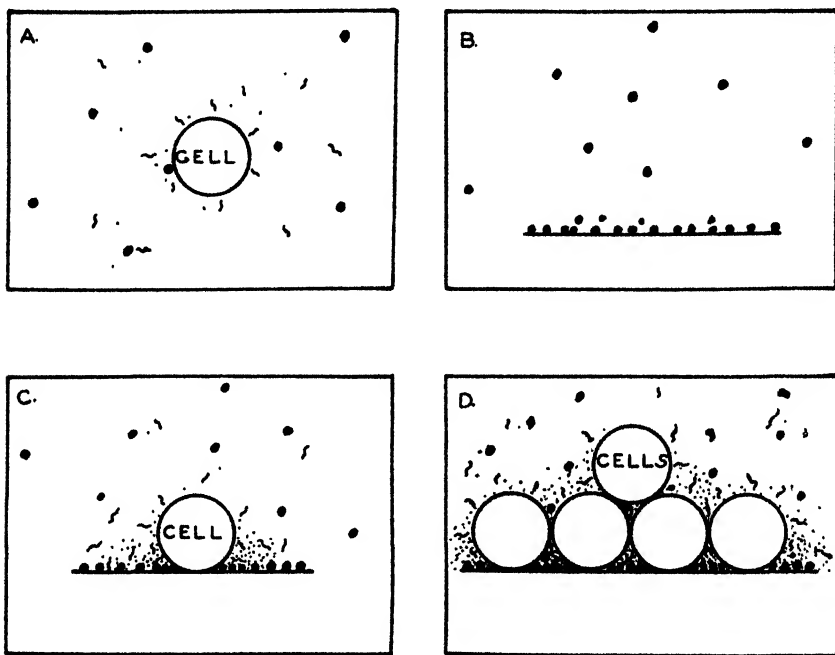


FIG. 2. A, A free-floating bacterial cell surrounded by a few suspended particles of food (dark circles) which must be hydrolyzed by the exoenzyme (helicoidal lines) before the resulting hydrolyzates (dots) can be ingested and assimilated. B, Particles of food concentrated in a monomolecular layer on a solid surface. C, Food particles are more available to the cell on the solid surface where the interstices at the tangent of the bacterial cell and the solid surface retard the diffusion of exoenzymes and hydrolyzates away from the cell. D, Multiple cells form additional interstitial spaces.

facilitate the orientation of bacterial exoenzymes in the most advantageous position (Danielli, 1937). According to Adam (1938), it has been found by Willstätter that adsorption of a soluble enzyme on a neutral inactive support will often increase its stability and activity.

From their studies on the relation of solid surfaces to bacterial activity, Heukelekian and Heller (1940) conclude that "Surfaces enable bacteria to develop in substrates otherwise too dilute for growth. Development takes place either as a bacterial slime or colonial growth attached to the surfaces. Once a biologically active slime is established on surfaces, the rate of biological reaction is greatly accelerated."

KINDS OF SOLID SURFACES

In order to appraise the effect of surface upon bacterial activity the solids used must be chemically inert. Glass has proved best for this purpose because, besides being virtually insoluble, it is available in a great variety of forms and shapes, and transparent glass slides lend themselves readily to the direct microscopic studies of attached materials. However, glass is by no means the only substance which can be used to demonstrate the attachment of bacteria and the growth-promoting properties of solid surfaces.

When submerged in the sea, bacteria attach to celluloid, cellophane, vinylite, lucite, bakelite and other plastics both transparent and opaque. There is a marked difference in the rate of attachment of bacteria to, the accumulation of organic matter on, and the growth-promoting properties of different kinds of plastics. Details can be given only after war-time restrictions are lifted because these studies may have direct bearing upon the "fouling" of ships' bottoms and other submerged surfaces (ZoBell, 1939). There is evidence that some plastics are attacked biochemically by bacteria.

Bacteria attach less readily to solid surfaces which are rendered hydrophobic by the application of greases, waxes and certain kinds of lacquers than to hydrophilic surfaces of low surface tension which are more wettable. Likewise positively charged surfaces are more conducive to the attachment of bacteria than negatively charged ones. Neither color, plane or angle of immersion nor the degree of roughness or smoothness of a surface seems to influence the attachment of bacteria. For unexplained reasons bacteria generally attach more readily near the edge of glass slides than in the center regardless of the position in which the slides are immersed. Bacteria attach to gold and silver surfaces but clean silver, copper, aluminum and iron surfaces appear to exert an adverse oligodynamic effect.

Porcelain surfaces also promote bacterial activity in dilute nutrient solutions, unglazed porcelain particularly. Ignited sand, asbestos fibres, emery grit and kieselguhr are likewise beneficial. It has been demonstrated that these substances adsorb organic matter from sea water, and proportionately many more bacteria are associated with the solid particles than are found in similar volumes of water. The bacteria are usually so tenaciously attached to the solid particles that oxygen consumption or similar tests must be used to demonstrate their presence and activity. Indifferent results have been obtained with bentonite, talc, kaolin and various samples of marine clays although they adsorb organic matter. This may be because particles smaller than bacteria adsorbed on bacteria may be inimical as suggested by the work of Peele (1936).

Conn and Conn (1940) found that bentonite, kaolinite, beidelite and illite stimulated bacterial activity, which they attributed to increased surface and other factors. Bigger and Nelson (1941) found that ignited asbestos, barium sulfide, barium hyposulfite, barium sulfite, tricalcium phosphate, ferric phosphate, ferrous silicate, kaolin, kieselguhr, magnesium silicate, manganese dioxide, permutit, silica, silver sand, soil, unglazed porcelain, talc and zirconium silicate

make it possible for coliform bacteria to grow in very dilute nutrient solutions (less than 0.4 mgm./l. of organic nutrient). Although some of the substances used by Bigger and Nelson may be active in other ways, they attribute the beneficial effect to the concentration of nutrients by the substances.

Studies with sand, kieselguhr and similar substances reveal that another factor is involved, namely, the distribution of the solid surface in the solution. For example, a layer of sand or kieselguhr 2 to 3 cm. thick in the bottom of a bottle accelerates bacterial activity little more than does a layer only 2 to 3 mm. thick, although the thicker layer presents ten times as much surface area, presumably because little more of the solution is in intimate contact with the sand in one case than in the other. In order to be most effective the solid surface must be distributed throughout the solution. This has been demonstrated by using different shapes of glass.

Enough 2 mm. glass beads were placed on the bottom of 145 ml. glass-stoppered bottles to provide the same surface area as provided by a dozen pieces of 6 mm. glass tubes standing upright in similar bottles. In the latter arrangement none of the water was more than 3 mm. from a solid surface whereas in bottles partly filled with glass beads much of the water was considerably more than 3 mm. from a solid surface. Bacteria consumed oxygen more rapidly in the bottles with the glass tubes than in those having a similar area of glass beads. In fact, the glass tubes stimulated bacterial activity more than a 2- to 3-cm. layer of silica sand in the bottom of the bottles. Although the latter presented much more solid surface than the glass tubes, it was not in close proximity to the water.

Glass wool has been used to provide large areas of solid surface throughout the bottles of water, but it has not proved to be entirely satisfactory for experimental purposes because it is difficult to wash free of impurities, and it is virtually impossible to free the glass wool of adherent gas bubbles.

According to Prescott and Winslow (1931), Kohn conducted experiments which suggested that the multiplication of bacteria during storage in glass receptacles is attributable partly to the solution of certain constituents from glass which favor bacterial life. While this may be true of certain waters, there is no evidence that the waters which we have examined dissolve any growth-promoting substance. The beneficial effects of surface observed with Pyrex glass have been comparable to those obtained with various kinds of soft glass. When stored at 0°C. in contact with large surface areas of different kinds of glass including glass wool and glass beads, the water is rendered less growth-promoting rather than more so which, as discussed above, is due to the adsorption of organic nutrients. That nothing toxic is dissolved from the glass is shown by the fact that such water regains its growth-promoting properties upon the addition of a fraction of a mgm./l. of glucose. It is not a reflection upon the work of Kohn to suppose that the growth-promoting constituents dissolved from glass were organic compounds because, as emphasized by Adam (1938), it is very difficult to prepare chemically clean glass surfaces. Soft glass is more difficult to clean than harder glasses, and significantly, Kohn noted the greatest

bacterial increases in bottles made of softer glasses. As noted by Esty and Cathcart (1921) unbuffered media have a tendency to become more acid during heat sterilization in Pyrex glass and more alkaline in soft glass tubes, but in all of our observations on the effect of glass surfaces we have used well buffered media.

DISCUSSION

Conn and Conn (1940) speculate that besides concentrating nutrients, solid surfaces may adsorb toxic products and increase the availability of oxygen. These two factors may be operative under certain conditions but it is doubtful if either factor helps to account for the beneficial effect of solid surfaces in extremely dilute solutions.

For many years more or less inert solids such as chalk, glass beads, glass wool, sand, kaolin, clinkers, sponge, animal tissue, charcoal, iron nails, cellulose, etc., have been employed in liquid media to promote the growth of bacteria (see literature cited by Breden and Buswell, 1933). Some of the substances probably provide a favorable oxygen tension and others may serve as a support for bacteria. Breden and Buswell (1933) point out that in continuous fermentation processes (vinegar, sewage beds, etc.), inert materials support organisms, "preventing their loss with removal of spent liquor and making possible the heavy inoculation of fresh substrates." They found that shredded asbestos improved methane fermentation. Numerous methane-producing organisms grew on the asbestos, small pieces of which could be used to inoculate successfully new batches of soluble substances. Butterfield (1937) attributed the beneficial effect of activated sludge to the adsorption of dissolved and colloidal materials.

Peele (1936) investigated several factors, including electric charges and base-exchange complex, which influence the adsorption of bacteria by soils. He found that the addition of soil to bacterial suspensions sometimes retarded the evolution of carbon dioxide, which he attributed to the adsorption of bacteria by soils. Similar results were obtained by Chudiakow (1926). Clay particles considerably smaller than bacteria predominated in the soils used by Peele and Chudiakow, so it is conceivable that such particles surrounding the bacteria retarded the assimilation of nutrients or other normal bacterial activities. McCalla's (1940) observation on the large number of active adsorption sites on a bacterial cell, which may have an affinity for minute positively-charged particles, supports such a concept. According to Dianowa and Woroschilowa (cited by Peele, 1936), "the decrease in biochemical activity when a portion of soil was shaken with a culture of bacteria was much greater when the soil was made up of very fine particles, such as silt and clay, than when the soil contained a larger proportion of sand." Also the adsorption of bacteria by soil probably caused them to settle to the bottom of the medium where they would be less active than when distributed throughout the medium. Peele used *Azotobacter chroococcum* as the test organism.

On the other hand Söhnngen (1913) records that the activities of hydrocarbon-oxidizing bacteria are accelerated by adsorption by soil. According to Rubent-

schik *et al.* (1936) the activity of some bacteria (nitrifiers) is retarded while that of others (sulfate reducers) is increased by adsorption by lake mud.

In their studies on the adsorption of bacteria by inert reagents, Gunnison and Marshall (1937) found no evidence that the beneficial clinical effects which sometimes follow the oral administration of kaolin, charcoal, Fuller's earth, etc., could be attributed to the inactivation of adsorbed pathogens. They believe that the adsorption of toxins or enzymes is more likely to account for the reported clinical improvements than the removal of bacteria by adsorption. In their experiments, like those of Peele (1936) and Chudiakow (1926) with soils, the particles used were mostly smaller than bacteria and the concentration of nutrients was high. In our experiments a beneficial effect of solid surface has been observed only in very dilute nutrient solutions and on solid surfaces which are large in comparison to the size of bacteria.

Further studies on the influence of particle size, food concentration and composition, the behavior of different kinds of bacteria, electric charge, surface tension, electrolytes, pH and other factors which influence the relation of bacteria to solid surfaces are indicated.

SUMMARY

Minute but demonstrable quantities of organic nutrients are adsorbed from sea water by glass.

In dilute nutrient solutions such as sea water the organic matter concentrated by adsorption on solid surfaces enhances bacterial activity.

Many of the bacteria found in sea water are sessile, growing exclusively or preferentially attached to a solid surface.

Most sessile bacteria appear to grow on solid surfaces by exuding a mucilaginous holdfast. A few have stalks. The exudate may aid in concentrating nutrients in the vicinity of the attached bacteria.

The beneficial effect of solid surfaces is usually evident only in very dilute nutrient solutions (less than 10 mgm./l). The beneficial effect is more pronounced with colloidal or poorly soluble substances than with those which are very soluble.

It is believed that besides concentrating nutrients by adsorption and providing a resting place for sessile bacteria, solid surfaces retard the diffusion of exoenzymes and hydrolyzates away from the cell thereby promoting the assimilation of nutrients which must be hydrolyzed extracellularly prior to ingestion.

Glass, plastics, porcelain, sand, kieselguhr and other inert particulate materials are surface active. Particles larger than bacteria are more beneficial than smaller particles. Particles smaller than bacteria may be harmful.

In order to be most effective the solid surface must be distributed throughout the dilute nutrient solution.

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ELECTRON MICROSCOPE STUDIES OF BACTERIAL VIRUSES¹

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Physico-chemical (Schlesinger, 1933; Northrop, 1938; Kalmanson and Bronfenbrenner, 1939) and biological (Ellis and Delbrück, 1939) studies of bacterial viruses (bacteriophages) in the last few years have led to a revival of interest in this group of viruses, particularly as a material on which one can study under very favorable conditions properties which may be common to all viruses. Ease and accuracy of titration and the possibility of working under biochemically controlled conditions make bacterial viruses an ideal object for such investigations.

The electron microscope, recently introduced as a tool for biological research, has been applied to the study of animal and plant viruses, and also of bacterial viruses (Ruska, 1940; Pfankuch and Kausche, 1940; Ruska, 1941; Luria and Anderson, 1942). Ruska (1941) published micrographs of suspensions of bacterial viruses, in which "sperm-shaped" particles can be seen. Ruska suggested that these particles should be interpreted either as the virus itself or as bacterial constituents.

In December 1941 and March 1942 Luria and Anderson (1942), through arrangements made with the National Research Council Committee on Biological Applications of the Electron Microscope, were enabled to study several bacterial viruses with the RCA electron microscope. They found sperm-shaped particles in the virus suspensions, and identified them as virus particles on the basis of considerations which will be more fully developed in the discussion of the present paper.

During the summer of 1942 the present authors availed themselves of the presence of the RCA electron microscope at the Marine Biological Laboratory, Woods Hole, to study the interaction of bacterial viruses with their bacterial hosts. Previous growth experiments (Delbrück and Luria, 1942) had served to analyze the various stages of the interaction (specific adsorption, latent period of virus multiplication, virus liberation and lysis of the bacterium). This analysis formed the basis for the interpretation of the results.

The present paper describes and discusses the results of this whole series of electron micrographic studies of bacterial viruses.

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MATERIAL AND METHODS

The RCA electron microscope and its mode of operation have previously been described (Anderson, 1942). A suspension of the material to be studied is deposited on a thin (10–20 $m\mu$) collodion membrane, which in turn is supported by a fine wire mesh screen holder. The suspension is left for a few minutes on the membrane in order to permit the particles to settle out and to adhere to the membrane. The holder is then “washed” by dipping it a few times into distilled water; the “washing” is necessary to remove salts, which otherwise crystallize and spoil the preparation.

Several strains of bacterial virus were studied using 60 kV electrons. Two of them, α and γ , received particular attention.⁴ In an earlier paper, the growth of these two viruses, which are active on the same host (*Escherichia coli* strain B), has been described in detail (Delbrück and Luria, 1942). Adsorption of virus α on a growing sensitive cell of strain B at 37°C. produces, after a latent period of 13–17 minutes, lysis of the cell with liberation of about 140 infective units of virus. For virus γ on the same host the latent period is 21–25 minutes; the number of infective units liberated is about 135 per cell.

Crude suspensions of the viruses of a titer between 5×10^9 and 2×10^{10} units/ml. were found to give good results in the electron microscope experiments. Suspensions of virus γ which had been partially purified by differential ultracentrifugation were also investigated.⁵

For the study of the interaction of virus and bacteria, an excess of virus was added to a young broth culture of bacteria under standard conditions. Samples were taken at various intervals with a small wire loop and deposited on specimen holders of the electron microscope. The specimen on the holder was incubated for a time in a moist chamber, so that the growth of the bacteria could continue under conditions similar to those in the broth culture. The holder was then washed in distilled water and allowed to dry rapidly in air; the process of washing and drying takes less than one minute. A specimen prepared in this manner will show the state of affairs in the growing mixture at a definite moment, which can be taken as that of washing. In some experiments, bacteria from a broth culture or from a young slant were mixed with a drop of virus suspension on the holder and, after incubation, were washed and dried as described above.

RESULTS

1. *The virus particles*


Micrographs of suspensions of virus α and of virus γ show the presence of particles of characteristic shape and size, specific for each strain (figs. 1, 2, 3). Regarding the identification of the particles visible on these pictures, with the

⁴ Virus α and virus γ , for which the authors are indebted to Dr. J. Bronfenbrenner, were originally designated P28 and PC. Virus γ = PC has been purified by Kalmanson and Bronfenbrenner (1939). The practical reasons for the change of name were given by Delbrück and Luria (1942).

⁵ One of the authors (S. E. L.) is greatly indebted to Dr. D. H. Moore for collaborating in the work of purification.

viruses rather than with inert bacterial constituents, we note: the two viruses were grown at the expense of the same host; the same bacterium then produces particles of type α if acted upon by virus α , and particles of type γ if acted upon by virus γ .

The particles of virus α have a round "head," 45–50 μ in diameter and uniformly dark in the micrographs, that means, uniformly scattering for 60 kV electrons. To this round "head" is attached a "tail," about 150 μ long and not more than 10–15 μ thick. The tail appears either straight or slightly curved.

The particles of virus γ present a very peculiar aspect. To an oval head, $65 \times 80 \mu$, a straight tail, 120 μ long and 20 μ thick, is attached at one of the narrow poles. The head always shows a structure consisting of light and dark areas. The structure, although striking enough to make the particles immediately recognizable, is quite variable. Four frequent configurations can be described schematically as X-shaped, Z-shaped, inverted Z-shaped, and diplococcus-shaped (). These various configurations can not all be accounted for by one three-dimensional structure seen under different angles. The interpretation of these structures will be discussed later.

The particles described above are never seen in suspensions without virus activity; their number is in direct proportion with the activity.

In suspensions of virus γ partially purified by differential centrifugation, the same particles are visible. However, many of them appear to be damaged; the tail is often broken, sometimes altogether missing. Since during the process of purification a large part of the activity had been lost, we believe that the abnormal particles visible in these suspensions have been mechanically damaged and inactivated.⁶

Particles of another coli virus are visible in fig. 16. They are round, 50–60 μ in diameter, and no tail can be seen. This of course does not preclude the existence of a tail, which might be too slender to be visible in the micrographs.

Particles of a staphylococcus virus⁷ are shown in fig. 4. They have a head about 100 μ in diameter, and a tail about 200 μ long.

2. The growth of bacterial viruses and lysis of the host

a. Virus γ . Figs. 5–12 are micrographs of samples taken from growing mixtures of virus γ and sensitive bacteria. Under the conditions of the experiment, infected cells yield, after a latent period of 21–25 minutes, an average number of 135 infective units of virus (Delbrück and Luria, 1942). Results from two such experiments will be given. The two experiments differed in the multiplicity of infection, *i.e.*, in the number of virus units available in the mixture for each bacterium, as given by plaque count assays.⁸ The first experiment was one of high multiplicity, each bacterium being infected on the average by eight virus units,

⁶ This suggests the opportunity of controlling on crude virus suspensions the results of electron micrographic studies of purified preparations of viruses in general.

⁷ Obtained through the courtesy of Dr. H. Zaytzeff-Jern.

⁸ The "multiplicity of infection" is defined as the ratio *adsorbed virus/bacteria* (Delbrück and Luria, 1942).

while in the second experiment the multiplicity of infection was between two and three.

Figs. 5-9 refer to the first of these experiments, in which the multiplicity of infection was high. Adsorption experiments (Delbrück and Luria, 1942) had shown that under these conditions practically all the virus is taken up by the bacteria in less than ten minutes, and that more than 99% of the bacteria are infected. Fig. 5 shows one of several micrographs of a sample that was dried after 15 minutes of contact between bacteria and virus (10 minutes in the test tube and five minutes on the specimen holder). At this stage, all bacteria appear normal in structure. Some particles of virus can be seen adsorbed on the edge of the bacterium, on or within the clear peripheral zone of the cell, which arises when the bacterial protoplasm shrinks away from the cell-wall during the process of drying. The existence of this clear zone enables one to see details on about one-third of the total surface of the bacterial cell-wall.

We obtained seven good pictures of bacteria from this sample, and on these seven bacteria, a total of 22 adsorbed virus particles could be seen in all, an average of about three particles per bacterium. Keeping in mind that only about one-third of the surface is in clear view, we can estimate that there are in reality about nine particles adsorbed per bacterium. Within the limit of this rather crude evaluation, we obtain, therefore, fair agreement between the number of particles attached to the surface of the bacterium, as revealed by the electron microscope, and the number of virus units attached to each bacterium, as inferred from plaque count assays. This agreement may be taken as further evidence for the identification of the visible particles with the virus, and as confirming the conclusion previously reached by indirect methods only (Delbrück and Luria, 1942), that infective titers obtained by plaque count correspond closely to the actual number of infective particles present in a suspension. We also note an unexpected and important fact, namely, that the adsorbed particles remain at the surface of the bacterium; at least this must be true for the majority of the adsorbed particles.

No free particles were visible on these micrographs. This is to be expected since, as pointed out above, adsorption is practically complete in ten minutes.

Growth experiments had indicated that lysis and virus liberation occur for virus γ after a minimum latent period of 21 minutes. Electron micrographs taken at 15 minutes confirm this by the absence of lysed bacteria. In contrast to this, micrographs of samples dried at 23 or 26 minutes reveal a completely different picture. By singular chance, we obtained from a series of unusually favorable fields, pictures of a number of bacteria caught in various phases of disintegration.

Figs. 6 and 7 show a long bacterium (not unusual in young broth cultures of this strain), one end of which has burst open and has liberated a flood of material in which several hundred particles of virus γ are visible. Along with the virus particles, a granular material has come out from the bacterium. These granules are of uniform size and are much smaller than the virus particles, being 10-15 $m\mu$ in diameter. The increasing transparency of the bacterium from the normal to the bursting end shows how the bacterial content is shed from the bursting

end. The diffuse mass lying across the bursting end of the bacterium probably contains also the remains of another lysed cell. It is most noteworthy that the bacterial contents show, besides the virus particles, no other particles of similar size. The dark spots on the cell-wall of the long bacterium are either single or groups of virus particles. It is impossible to say whether they are inside or outside of the cell-wall.

It will be seen in figs. 6 and 7 that those parts where the two fields overlap agree in the finest details. Since these two figures resulted from separate exposures, they show that the objects imaged are not noticeably altered by the very intense electron irradiation necessary for focusing.

The cells shown in figs. 8 and 9 appear to be in a later stage of lysis. They are "ghosts," empty cell-walls from which all content has been liberated, and are surrounded by virus particles and protoplasmic granules. Holes of various sizes are visible in these bacterial cell-walls. Virus particles in large number (80 in one case, 150 in the other) surround the empty cell-walls. Their location in the immediate vicinity of the cells suggests that lysis has taken place after the specimen had been washed, *i.e.*, during the brief period of drying. Therefore, the number of visible particles should and does correspond to the average yield of virus per bacterium, as determined by growth experiments, namely 135.

Side by side with the lysed bacteria we find in these specimens bacteria which are not yet lysed. They show on their edge many more adsorbed virus particles than those from specimens dried at 15 minutes, when lysis had not yet started. These virus particles must have been adsorbed after their liberation from neighboring lysed bacteria. Since practically all bacteria in the specimen had become infected in the first minutes of the experiment, these bacteria must also be close to lysis. We conclude that the ability to adsorb virus remains unimpaired until very close to the moment of lysis. This conclusion had been reached previously on the basis of growth experiments (Delbrück, 1940). Fig. 10 shows the protoplasmic granular material at a higher magnification.

In the second experiment mentioned above, in which the multiplicity of infection was only two or three, micrographs of specimens dried at 5, 10, and 15 minutes show bacteria of normal aspect. No adsorbed virus particles are visible, corresponding to the low multiplicity of infection. However, the bacteria are infected; in specimens dried at 25 minutes most bacteria are seen to be lysed, and the remaining ones show numerous virus particles adsorbed on their surface. These bacteria appear to be damaged and near to lysis (fig. 11).

b. Virus α . The interaction between the same strain of bacteria and virus α was studied in an experiment in which the multiplicity of infection was about five. In specimens prepared at five or ten minutes, the bacteria appear normal, occasionally with one or a few adsorbed virus particles visible on their edge. At fifteen minutes (figs. 13 and 14) we witness the lysis of bacteria, as expected on the basis of growth experiments, which give a latent period of 13–17 minutes for the lysis produced by virus α in this host. Lysis is accompanied by the liberation of particles of the type characteristic for this virus. Cells in the process of lysis and "ghosts" are both considerably swollen.

The material which is liberated from the lysed cell along with the virus particles

is again granular, the granules being of the same size as in the case of lysis produced by virus γ (fig. 12). This is strong support of the interpretation of these minute particles as constituents of the bacterial protoplasm.

All bacteria which are not lysed within 15 minutes have several virus particles adsorbed (fig. 15); these obviously come from the lysed cells. The bacterium in fig. 15 has almost completely divided, but the cell-walls of the two cells are still connected by an X-shaped bridge.

c. Other viruses. Figs. 16 and 17 illustrate the action of still another virus which is active on a different host, a motile strain of *E. coli*. This virus was studied extensively several years ago by one of the authors (Delbrück, 1940). The bacterium in fig. 16 shows flagella and adsorbed virus particles. Fig. 17 shows an empty cell-wall after lysis. It is clearly visible that the flagella have remained intact. The same can be said of the case of a virus active on *Salmonella sp.* (Poona type)⁹ (fig. 18). In these two cases at least, the flagella do not appear to be damaged by the action of the virus.

DISCUSSION

There can hardly be any doubt that the sperm-shaped particles in suspensions of bacterial viruses are the particles of virus. They are present in amounts proportional to the activity and they are never present in suspensions without virus activity. The structure of the visible particles is specific for each strain and a bacterium liberates the particles which are characteristic for the virus which has acted upon it. The use of two different and unrelated viruses acting on the same host eliminates the possibility that the particles might be natural protoplasmic components of the bacterium.

The behavior of the visible particles during the reproduction cycle of the virus, showing their specific adsorption and their liberation in the expected amount after the expected latent period, offers further reasons for their identification.

Size and shape of the particles deserve special attention. The size of the "head" is close to that which had previously been inferred for various bacterial viruses by indirect methods. For viruses α and γ the sensitivity to irradiation with x-rays has been tested quantitatively (Luria and Exner, 1941). From these data the sensitive volume, *i.e.*, the volume within which absorption of energy from the x-rays leads to inactivation of the virus has been calculated. This sensitive volume may be smaller than the true volume. The irradiation data therefore give minimum values for the particle sizes. The results were 36 $m\mu$ for virus α and 50 $m\mu$ for virus γ , *i.e.*, in both cases about 30% smaller than the sizes given by the electron microscope pictures. We conclude that irradiation experiments do give values close to the real ones, and give correct values for the relative sizes of different viruses.

Kalmanson and Bronfenbrenner (1939) have studied the diffusion of virus γ purified by differential filtration. They find that their virus suspensions contain

⁹ Obtained through the courtesy of Dr. M. L. Rakieten.

two fractions with different rates of diffusion. The larger fraction diffuses at a rate corresponding to a particle diameter of 16–18 $m\mu$. A very small fraction diffuses faster, corresponding to a particle size of 3–4 $m\mu$. These authors assume that the small particles are usually attached to larger unspecific carriers (the slower diffusing particles), and that they occasionally become free, still retaining their activity. The electron microscope pictures do not support this view, since the particles visible on these pictures are too regular in size and structure to be interpreted as bacterial debris to which the virus might be adsorbed. The electron microscope could not show particles having diameters of 3–4 $m\mu$. But if such small virus particles existed, they should show up in irradiation experiments as a very resistant fraction. Careful search for such an x-ray resistant fraction of virus has given completely negative results (Luria and Exner, 1941).

Size determinations of the strain of staphylococcus virus shown in fig. 4 have not been made by any other method. However, the filtration and centrifugation studies of Elford and others have shown that different strains of staphylococcus virus differ little in size, in contrast to the viruses of the coli-dysentery group. It may therefore be permissible to compare our values with those obtained for other staphylococcus viruses by other methods. Irradiation data have given 50 $m\mu$, somewhat lower than the value here obtained (100 $m\mu$). Ultrafiltration studies gave values between 50 and 78 $m\mu$, in fair agreement with the electron microscope value. Northrop (1938) has made extensive studies with purified preparations of a staphylococcus virus. From the rate of sedimentation in the ultracentrifuge a value of 60–90 $m\mu$ (mol. weight 3×10^8) was obtained, again in good agreement with our value. Diffusion experiments on Northrop's virus gave results which depended on the concentration of virus. In concentrated suspensions the diffusion rate corresponded to a molecular weight of about 3×10^8 , in agreement with the centrifugation value. However, in highly diluted suspensions the rate of diffusion was found to be faster, corresponding to a particle size of about 10 $m\mu$. In explanation Northrop proposed a reversible equilibrium between small particles and large particles. If this were true, plaque count assays should give titers corresponding to the number of small virus particles, since these assays are done at extreme dilution. The titer of large particles which are presumed to be present at high concentrations should then be almost a thousand times smaller than the plaque count titer. In contrast with this, we find approximate quantitative agreement between the plaque count titer and the number of particles visible on the electron microscope pictures. Therefore, Northrop's idea is not applicable to our cases.

Shape and structure of the virus particles, as revealed by the electron microscope, deserve special consideration. The pictures here reproduced give the impression of a somewhat more complex organization than the pictures of plant viruses (Stanley and Anderson, 1941) had indicated. These had shown either straight rods or round particles. Of the animal viruses, influenza virus showed very small, round particles (Chambers and Henle, 1941). Papilloma virus (Sharp, Taylor, Beard, and Beard, 1942a) and equine encephalomyelitis virus (Sharp, Taylor, Beard, and Beard, 1942b) have both round particles, but while

the particles of papilloma appear to be homogeneous, those of encephalomyelitis virus show an internal structure. The majority of the much larger bodies of vaccinia virus (Green, Anderson, and Smadel, 1942) show five granules of more scattering material.

The structure in the head of virus γ cannot be ascribed to the presence of material of higher specific scattering power. The most likely material could be phosphorus, which, per atom, should scatter about four times more strongly than carbon, nitrogen or oxygen. In nucleic acid, for instance, there is one phosphorus atom to 20 of the lighter atoms. Pure nucleic acid would therefore scatter only about 20% more than a similar compound without phosphorus. Since we can hardly assume that the dark regions are composed exclusively of compounds of such relatively high phosphorus content, the maximum contrast due to differences in specific scattering power could not be more than a few percent and could not show up in our pictures. We believe therefore that the dark parts represent regions of greater thickness. It is possible that the particles in the native state are oval, but upon drying the more aqueous parts collapse while the solid parts retain more scattering material, which forms the dark areas of the head. The images obtained therefore indicate that the distribution of solid material in the head of the particle of virus γ is not uniform. A detailed analysis of the structure visible in the heads of these particles will be given elsewhere.

A word may be added regarding the tendency to speak of viruses as molecules. This tendency received its greatest momentum from Stanley's discovery in 1935 that paracrystals of tobacco mosaic virus could be obtained by simple methods, and from the great number of subsequent studies of this and of other viruses along the lines of protein chemistry. Also the electron microscope pictures of plant viruses, revealing simple rods and spheres, seemed to encourage such a tendency. While it is true that no strain of bacterial virus has yet been crystallized, chemical studies on purified preparations have indicated that chemically, bacterial and other viruses are closely related (Northrop, 1938). It is only natural that chemical and physical studies of viruses have led scientists to the habit of thinking of viruses in terms of molecules. However, one should keep in mind that the concept "molecule" is flexible when applied to structures such as viruses. When we speak of a long chain compound as a molecule, neither its configuration nor even its composition is to be taken as necessarily definite. The ambiguities will be multiplied if still more complicated structures are considered, the parts of which are not all connected by primary valencies. Such "molecules" will share with living things the impossibility of delimiting unambiguously which atoms belong to them and which do not. While no harm is done by calling viruses "molecules", such a terminology should not prejudice our views regarding the biological status of the viruses, which has yet to be elucidated.

The study of the interaction of virus α and virus γ with their host has confirmed in every detail the picture of the process which had been deduced on the basis of growth experiments. As d'Herelle (1926) had early suggested, and as quantitative studies had shown (Delbrück, 1942), the infection of a bacterium, after a latent period characteristic of each virus, is followed by lysis of the bac-

terium with liberation of a large number of new virus particles. Quantitatively, too, the agreement between electron microscopic and growth experiments is excellent, both regarding the length of the latent period and regarding the amount of virus liberated by the bacteria. Several points, however, receive further light from the electron micrographs.

After lysis of a bacterium a cell-wall remains which, in contrast to those obtained from intense sonic vibrations (Mudd, Polevitzky, Anderson, and Chambers, 1941), is always more or less damaged and variously lacerated. The new virus is liberated from the interior of the bacterium. The pictures, however, give no indication in which part of the bacterium the virus is produced, whether in the deep interior of the cell or close to the inner surface of the cell-wall.

The pictures of lysed bacteria show, besides the particles of virus, also granular material of very regular units, 10–15 $m\mu$ in diameter. If these are to be interpreted as molecules, their size corresponds to a molecular weight of the order of 10^6 . These particles are liberated from the cell in great abundance, and seem to constitute the bulk of its protoplasm. The absence among the bacterial components of elements of size comparable to that of the virus particles explains why the latter can be studied so favorably in crude suspensions. It also explains the success of work on the purification of bacterial viruses by means of differential centrifugation and by filtration, and should encourage further work along these lines.

In a series of papers Krueger (1938) has proposed the idea that the bacterial cell contains a precursor of the virus particle, which, upon infection of the cell with a virus particle, is promptly converted into virus. This theory was elaborated as an analogue to the well-known relations between proteolytic enzymes and their precursors. According to this theory an uninfected bacterium of the strain here considered should contain on the average 140 precursor particles of virus α and 135 precursor particles of virus γ . The pictures show clearly that this is not the case, since bacteria lysed under the influence of virus γ show no evidence of particles resembling virus α and vice versa.

Finally, a point may be mentioned which seems to us perhaps of the greatest consequence. We have seen that the new virus is liberated from within the cell. On the other hand, the pictures of bacteria infected with virus γ and taken at fifteen minutes showed that the adsorbed virus particles, or at least most of them, do not penetrate into the interior of the cell but remain on the outer surface of the cell-wall. This observation creates a difficulty in interpreting virus growth. How do the infecting particles reproduce if they remain outside while the new virus is generated in the interior of the cell? One might assume either that the infecting particles act through the cell-wall, or that only one particle can enter the cell. The latter idea seems attractive in the light of results of growth experiments on multiple and mixed infection. These have shown that a bacterium always reacts as though only one particle of virus had been effective. The pictures here reproduced, if interpreted on the assumption that one virus particle enters the cell, would indicate that the entry of one virus particle bars the entry of other virus particles by making the bacterial cell-wall impermeable to them. The highly

peculiar phenomenon of mutual exclusion between virus particles attacking a cell would thus be explained by a mechanism alternative to that proposed in a previous discussion (Delbrück and Luria, 1942). An interpretation of this kind, for the correctness of which the experiments offer as yet hardly more than a hint, would suggest an analogy with the fecundation of monospermic eggs, and would lend support to those theories of the systematic position of virus which consider it as related to the host rather than as a parasite (*cf.* Hadley, 1928).

SUMMARY

1. Four strains of bacterial viruses have been studied with the electron microscope. In all cases the particles of virus could be identified on the micrographs. Three of these strains show "sperm-shaped" particles, consisting of a head and a tail. For the fourth strain, a tail is not visible on the micrographs. The particles of one of these viruses show a distinct structure in the head. The particle sizes agree well with the sizes inferred by some of the indirect methods.

2. The interaction between the virus and its host has been studied in detail in the case of two viruses which act upon the same strain of *Escherichia coli*. The micrographs demonstrate the adsorption of virus on the host and, after the predicted time, the lysis of the host with the liberation of virus particles of the infecting type. There is quantitative agreement between the numbers of particles visible on the micrographs and the numbers predicted on the basis of growth experiments for which plaque count assays were used. Along with the virus particles, the lysing cells shed protoplasmic material of uniform granular structure. The size of these granules is much smaller than that of the viruses and is independent of the virus under whose influence the bacterium is lysed.

3. Upon lysis the virus particles are liberated from the interior of the bacterial cell, for they are not visible on its surface up to the moment of lysis. In cases of multiple infection, the *infecting* particles of virus, or at least the majority of them seem not to enter the cell but to remain attached to the outside of the bacterial cell-wall.

4. The bearing of these results on the problems of the nature of viruses and of their systematic position is discussed.

We are grateful to Miss Nina Zworykin for taking many of the electron micrographs shown.

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PLATE 1

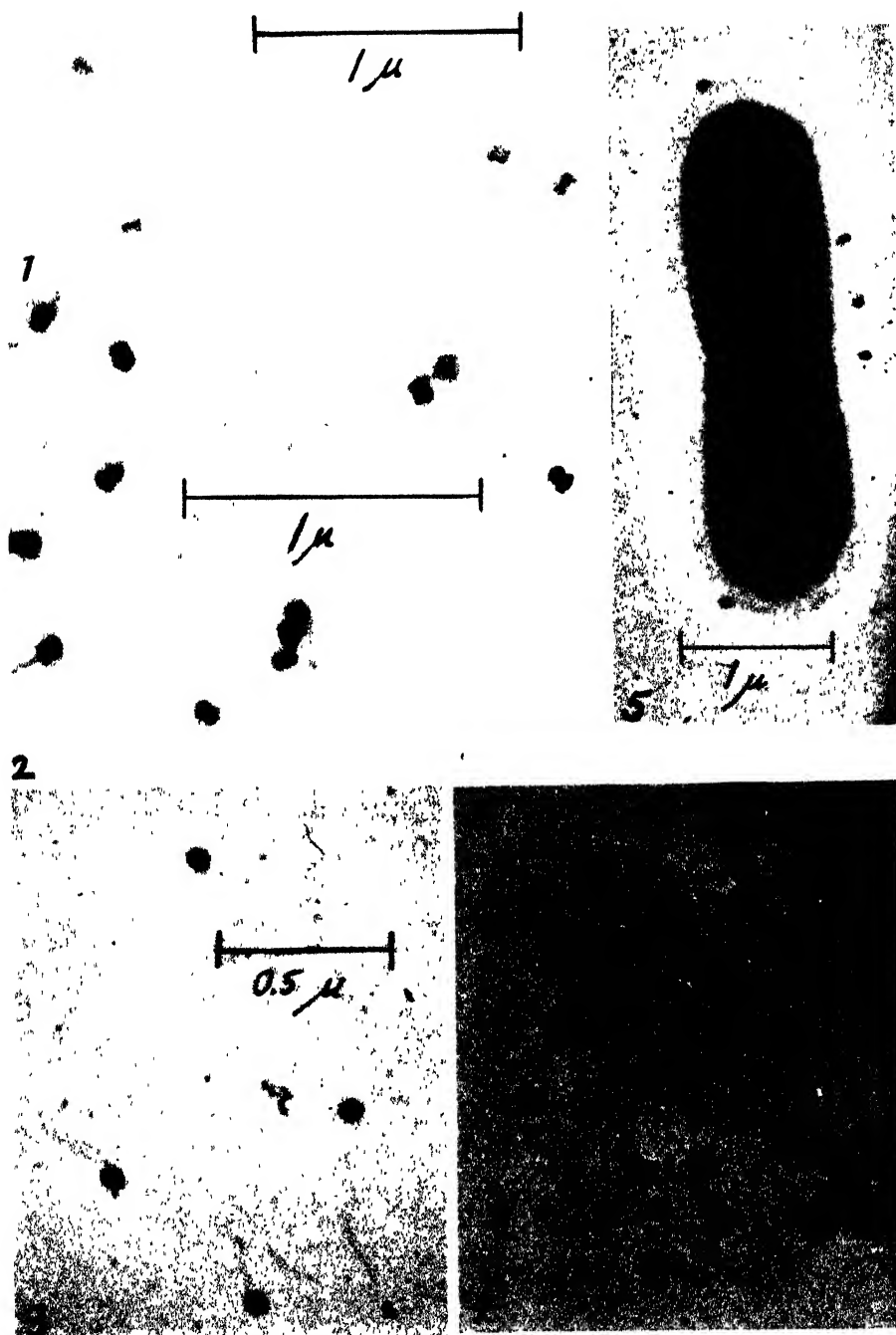
FIG. 1. Particles of virus γ . $\times 36,000$.

FIG. 2. Particles of virus γ . $\times 40,000$.

FIG. 3. Particles of virus α . $\times 47,000$.

FIG. 4. Particles of staphylococcus virus. $\times 20,000$.

FIG. 5. *E. coli* + virus γ . 15 minutes contact. A bacterium with adsorbed particles of virus. $\times 20,000$.



(S. E. Luria, M. Delbrück and T. F. Anderson: Electron Microscope Studies of Viruses)

PLATE 2

FIGS. 6 and 7. *E. coli* + virus γ . 23 minutes contact. A bacterium immediately after bursting, protoplasmic granules and several hundred particles of virus. The fields of the two pictures overlap in part. $\times 12,500$.



(N. E. Luria, M. Dellbrück and T. F. Anderson; Electron Microscope Studies of Viruses)

PLATE 3

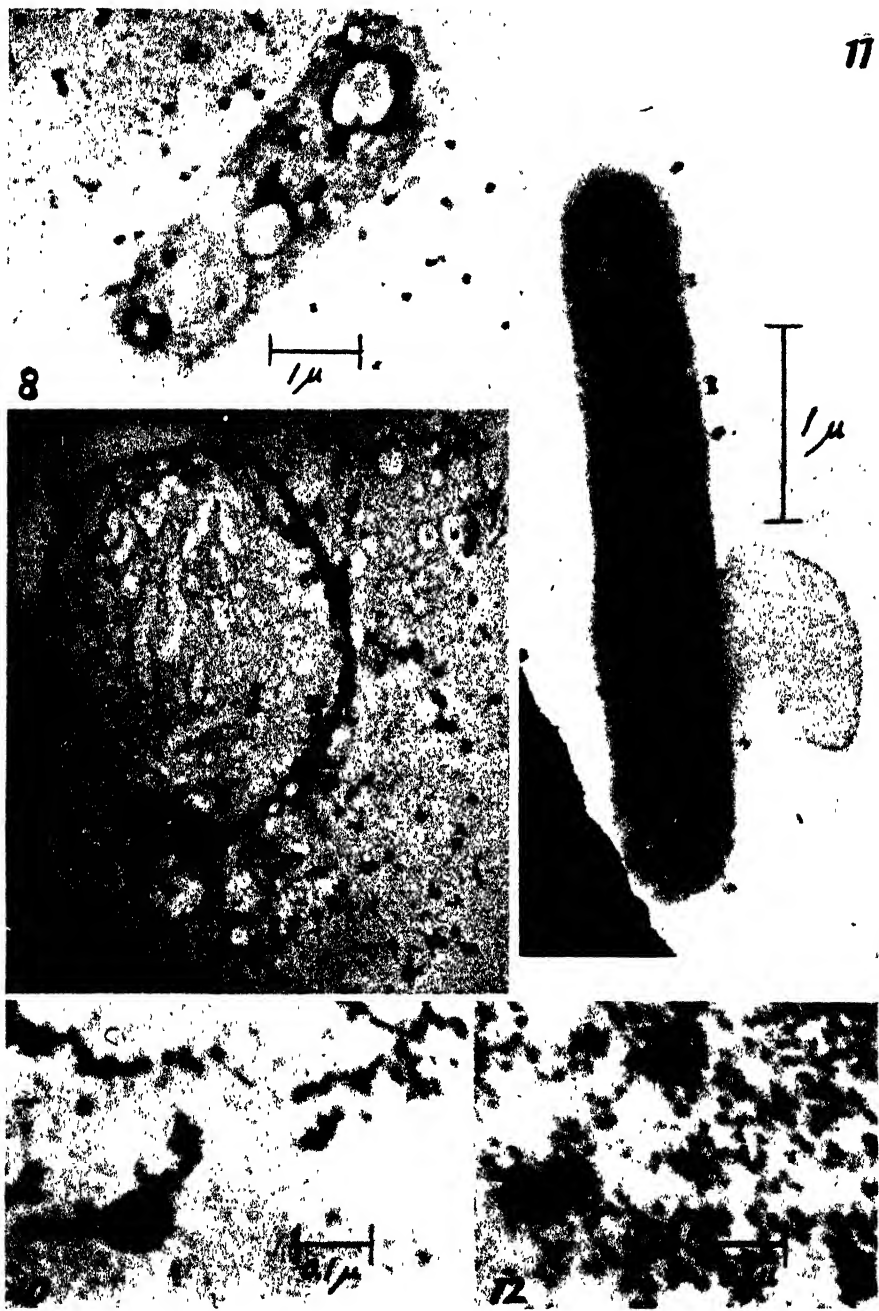
FIG. 8. *E. coli* + virus γ . 23 minutes contact. "Ghost" of a lysed bacterium $\times 12,500$.

FIG. 9. *E. coli* + virus γ . 23 minutes contact. Another "ghost". $\times 11,500$.

FIG. 10. *E. coli* + virus γ . 25 minutes contact. Detail of contents shed from a lysed cell, showing protoplasmic granules and one particle of virus γ . $\times 95,000$

FIG. 11. *E. coli* + virus γ . 25 minutes contact. A bacterium with ten adsorbed particles of virus visible on edges. $\times 27,000$.

FIG. 12. *E. coli* + virus α . 15 minutes contact. Detail of contents shed from a lysed cell, showing protoplasmic granules and three particles of virus α . $\times 100,000$.



(S. E. Luria, M. Delbruck and T. F. Anderson: Electron Microscope Studies of Viruses)

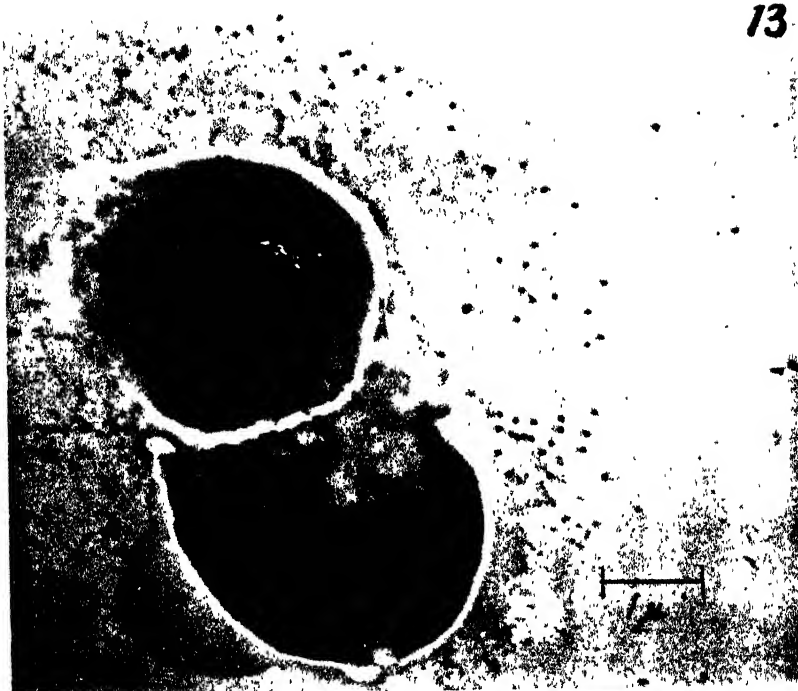
PLATE 4

FIG. 13. *E. coli* + virus α . 15 minutes contact. Lysis of a bacterial cell. Protoplasmic material and 93 particles of virus. $\times 13,500$.

FIG. 14. *E. coli* + virus α . 15 minutes contact. "Ghost" of a lysed bacterium, several particles of virus α . $\times 15,500$.

FIG. 15. *E. coli* + virus α . 15 minutes contact. Dividing bacterium with 19 adsorbed particles of virus visible on its edge. $\times 16,500$.

13



14



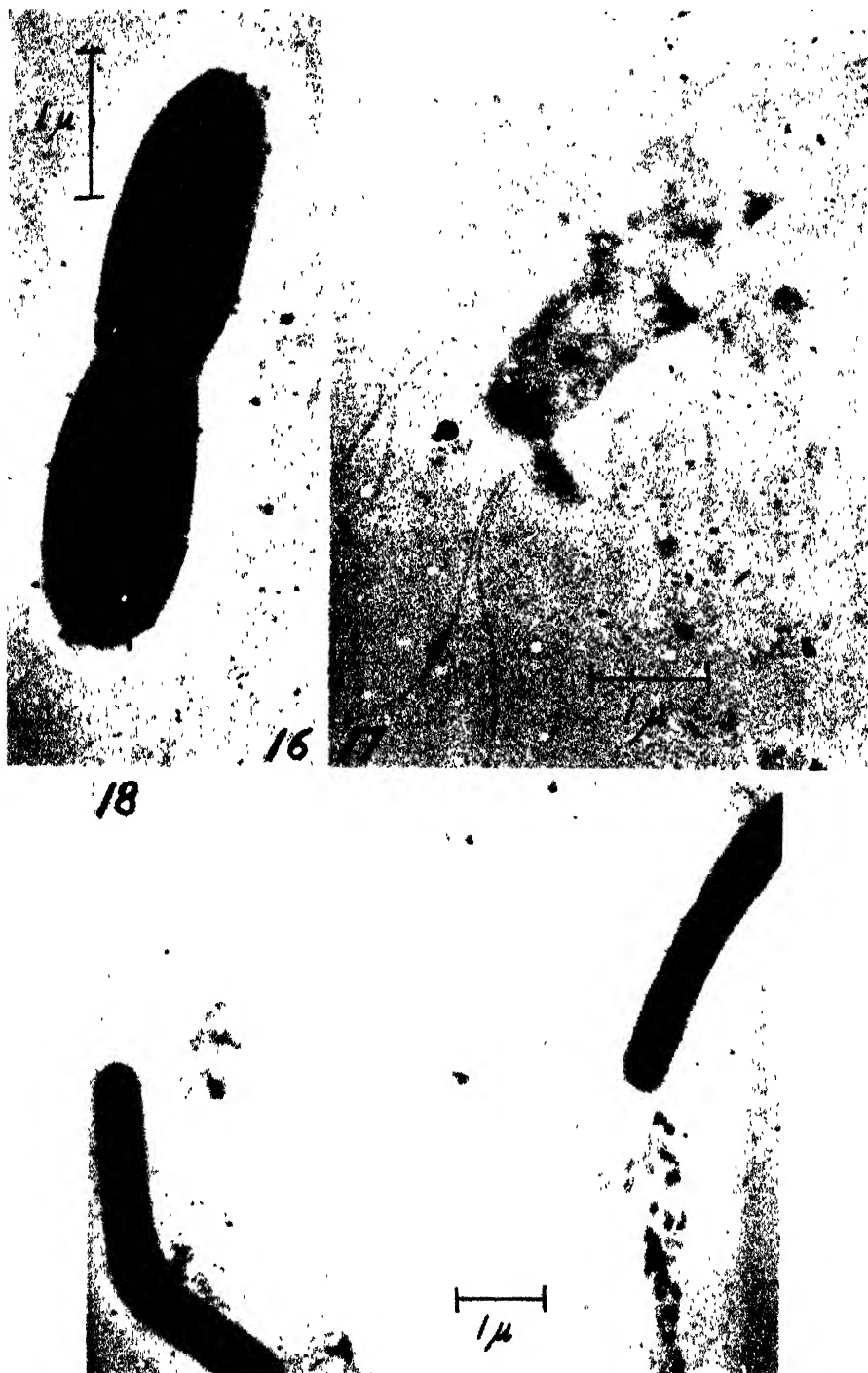
(S. E. Luria, M. Delbrück and T. F. Anderson: Electron Microscope Studies of Viruses)

PLATE 5

FIG. 16. *E. coli* (motile strain) + virus P₂. 22 minutes contact. Bacterium with 12 adsorbed particles of virus. 2 free particles of virus. $\times 19,000$.

FIG. 17. *E. coli* (motile strain) + virus P₂. 22 minutes contact. Lysed bacterium with flagella. $\times 16,500$.

FIG. 18. *Salmonella sp.* (Poona type) + virus SP. Centrifugation sediment of an unfiltered lysate. Lysed cells and two apparently normal cells. Flagella. $\times 12,000$.



(S. E. Luria, M. Delbruck and T. F. Anderson, Electron Microscope Studies of Viruses)

THE IDENTITY OF BACTERIUM COLUMBENSIS CASTELLANI

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The organism originally isolated by Castellani (1914) from "columbensis fever" and later (Castellani, 1938) assigned by him to the genus *Salmonella*, has been infrequently reported in the Western Hemisphere. The only records that have been located are a note reporting Taylor's (1924) isolation of what is now strain 595 of the American Type Culture Collection, the record in the catalogue of that collection mentioning the isolation by Shaw of their strain 4298, and an indirect reference by Jordan and McBroom (1934) to the occurrence of the organism in Panama.

The organism has been encountered in Ceylon, in the tropics of Asia and Africa, in the Balkans, and also in London, and reported in the papers of Fernando (1935), Fulle (1915), Giugni and Pistoni (1936), Guidetti (1937), Jacono (1921), Kelaart (1925), Lurie (1916), Marmo (1922), Scotti (1936, 1939), Spaar (1915), and Thomas (1936).

The fifth edition of Bergey's (1939) Manual lists *Salmonella columbensis* in the appendix to that genus, without a statement of its characteristics. It is explained that the species in that list are placed there because "the relationships of many of these are not clear." Certainly the characteristics recorded by Castellani and Chalmers (1919), by Castellani (1938), and in the earlier editions of the Bergey Manual, do not place the species within the genus *Salmonella* as at present defined.

A group of cultures has been studied in this laboratory which seem to be strains of this little-known species. Their comparison with the strains from the American Type Culture Collection and with the published descriptions, permits a restatement of the species characteristics and provides an opportunity to consider its generic position.

EXPERIMENTAL

A study has been made of 18 strains, including 4 which may be considered representative of the species since they came from the American Type Culture Collection. The origin of the cultures is indicated in table 1. They form a fairly homogeneous group having the following characteristics: Short rods, gram-negative, motile or nonmotile. Imvic ++ --. Gelatin-negative, sulfide-negative, tartrate-agar-negative, urea not decomposed. Litmus milk slowly made alkaline. Gas produced from carbohydrate. The following carbohydrates are fermented by all 18 strains: arabinose, galactose, glucose, levulose, mannitol, mannose, rhamnose, and trehalose. None of the strains fermented cellobiose, dextrin, inositol, inulin, lactose, alphanethylglucoside, raffinose, starch, or sucrose. Aesculin and dulcitol were fermented by approx-

imately half the strains. Certain other carbohydrates were usually fermented: 16 of the 18 strains fermented glycerol, 15 fermented maltose, 15 fermented salicin, 17 fermented sorbitol, and 14 fermented xylose.

None of the strains was agglutinated by serums available at the Salmonella Typing Center at Baylor College of Medicine. A rabbit was immunized by intravenous injection of a young broth culture of strain "teh" preserved with 0.5 per cent formalin. The serum titers with the homologous antigen were: H, 12,800; O, 6,400. This serum did not agglutinate live broth cultures, nor formalinized nor boiled suspensions, of any of the other strains.

The cultures were pathogenic for mice on intraabdominal injection, if the dose was large.

TABLE 1
Origin of strains

4	1928	ATCC #4208
7	?	ATCC #7278
46	1941	gastroenteritis, Dallas
226	1941	normal stool, Dallas
330	1942	gastroenteritis, Fort Sam Houston
377	1942	gastroenteritis, Fort Sam Houston
381	1942	gastroenteritis, Fort Sam Houston
595	1924	ATCC #595
747	?	ATCC #747
36025	1941	diarrhea, Austin
a	?	old stock strain
ash	1934	gastroenteritis, Providence
bradshaw	1942	normal stool, Dallas
j	1942	gastroenteritis, Dallas
jones 2	1942	gastroenteritis, Dallas
pscol	1941	gastroenteritis, Dallas
starr	1942	gastroenteritis, Dallas
teh	1942	stool of marasmic child, Dallas

DISCUSSION

The strains here described appear to form a definite group. They resemble the genus *Salmonella* but are indole-positive, usually salicin-positive, and do not give a positive sulfide test. Their characteristics differ sufficiently to be significant, from those set down by Kauffmann (1941, p. 175) as designating "probable *Salmonella*."

There seem to be valid differences also between these strains and the members of the genus *Proteus*. The strains here reported are not proteolytic, do not decompose urea, do not ferment sucrose, and are not "Hauch" formers on moist agar.

In some respects they resemble "paracolon" bacilli. This was the opinion also of Jordan and McBroom (1934). The reactions of some slow-lactose-fermenting "paracolon" cultures do in general resemble those set forth above. Castellani (1938) reported that his *S. columbensis* strains might ferment lactose,

especially when freshly isolated. Topley and Wilson (1936, p. 536) tabulate "*Bacterium columbense*" as possibly a slow lactose fermenter. Most authors have reported their strains lactose negative. None of the strains in the present study fermented either 0.5 per cent or 5.0 per cent lactose during 10 days incubation.

With the exceptions of motility and the fermentation of aesculin and dulcitol, these 18 strains form a group sufficiently uniform to represent a species. Castellani set up a species, *S. pseudocolumbensis*, for the nonmotile strains, but this hardly seems admissible. Neither the differences in aesculin and dulcitol, nor the failure of small numbers of the strains to ferment glycerol or sorbitol or xylose appears to furnish sufficient grounds for dividing the group. These considerations lead to the conclusion that these strains represent a quite definite species, for which the name proposed by Castellani is satisfactory enough.

There is at present no definite place for the classification of gram-negative bacilli that are lactose-negative, sucrose-negative, and indole-positive. It will be recalled that there is another better known species which has these characteristics. This is *Proteus morganii*. The "Morgan bacillus" is restricted in fermentative activity, producing acid and gas from galactose, glucose, glycerol (slowly), levulose and maltose; no other carbohydrate is attacked. Urea is decomposed slowly and weakly. Agglutination tests have not revealed any common antigens in *P. morganii* and *S. columbensis*. The other reactions of *P. morganii*, such as the Imvic, gelatin, sulfide, and litmus milk tests, are the same as those of *S. columbensis*.

Like *S. columbensis*, *P. morganii* was at one time assigned to the genus *Salmonella*. Its position in the genus *Proteus* is equivocal, and there are a number of points of similarity between it and *S. columbensis*, not only in its bacteriology, but in its habitat and in its somewhat vague relation to mild gastrointestinal disease in man.

It would seem advisable to examine the possibility of setting up a genus for Castellani's bacillus of columbensis fever, and possibly for Morgan's bacillus No. I as well. It is suggested that the name *Morganella* be coined, since the term *Castellanus* has been utilized previously in a different sense (Cerruti, 1930). *Morganella* could be defined as a genus of Enterobacteriaceae, tribe Salmonellae failing to ferment either lactose or sucrose, forming gas, and producing indole. The known species are *M. morganii* and *M. columbensis*.

Although Castellani isolated his organism as early as 1905, no description seems to have been published before 1914. The type species would therefore be *M. morganii* Morgan (1906).

SUMMARY

A description is given of a group of 18 strains which seem to belong to the species originally described by Castellani as *Bacterium columbense*. It is suggested that this species be grouped with *Proteus morganii* in a new genus to be called *Morganella*.

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FORMATION OF TYROTHRICIN IN SUBMERGED CULTURES OF *BACILLUS BREVIS*¹

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Since the isolation of tyrothricin from cultures of *Bacillus brevis* (Dubos, 1939), numerous papers have appeared dealing with the chemical and biological properties of this bactericidal agent and its components, gramicidin and tyrocidine (Hotchkiss and Dubos, 1940; Dubos and Hotchkiss, 1941; Tishler, Stokes, Trenner and Conn, 1941; Christensen, Edwards and Piersma, 1941; Lipmann, Hotchkiss and Dubos, 1941; Heilman and Herrell, 1941; Henle and Zittle, 1941; Little, Dubos and Hotchkiss, 1941; Robinson and Molitor, 1942). However, little is known of the physiology of the bacillus from which the bactericidal agent is obtained. The present investigation is concerned with the relation of the nitrogen nutrition of *B. brevis* to tyrothricin formation in stationary and submerged cultures. A method suitable for large scale production of tyrothricin in submerged cultures is described.

METHODS

The BG strain of *Bacillus brevis* was used exclusively.² The temperature and growth period were 37° and approximately 66 hours, respectively.

Qualitative tests for tyrothricin were made by adding 1 ml. of the supernatant fluid of a centrifuged culture of *B. brevis* to an equal volume of a 5-hour broth culture of *Micrococcus conglomeratus* (Strain MY) and incubating at 37° for 2 hours. Lysis of the micrococcus, as evidenced by clearing of the tube, indicates the presence of the bactericidal agent. This test is rapid and reliable; tyrothricin has never been isolated from a culture which failed to give a positive test for lysis.

Tyrothricin was determined, quantitatively, by the isolation procedure of Dubos and Hotchkiss (1941) modified to include ether extraction of the alcohol-extracted crude tyrothricin prior to precipitation with NaCl. Also, a more rapid method was developed which does not involve isolation of tyrothricin and is applicable to small amounts of culture. Cell material from an aliquot of a culture is collected by centrifugation and suspended in an amount of ethanol equivalent to the volume of the aliquot used. After 24 hours at room temperature, the alcoholic extract is diluted with water and the smallest amount necessary to cause complete lysis of the micrococcus culture is determined as described above. Usually about 4γ of tyrothricin are required for lysis. Comparative experiments

¹ This paper was presented at the joint meeting of the New Jersey and New York sections of the Society of American Bacteriologists in New York City, December 29, 1942 (*J. Bact.*, **45**, 29-30, 1943).

² We are indebted to Dr. Dubos for this culture.

have shown that the tyrothricin values obtained by this method correspond closely to the quantities that can be isolated from the same cultures.

EXPERIMENTS WITH COMPLEX NITROGENOUS MEDIA

Tyrothricin is readily formed by *B. brevis* in shallow stationary cultures (1 inch or less in depth) of hydrolyzed casein, gelatin, peptone or tryptone medium. However, this is not the case in aerated submerged cultures. A large number of experiments were made with 5-liter batches of tryptone medium in a 2-gallon iron fermenter, in which the rate of stirring, aeration (air), temperature and concentration of tryptone were successively varied. Although luxuriant growth of the bacillus occurred, no tyrothricin was formed. Also, cultures grown in 1-liter Erlenmeyer flasks containing 700 ml. of medium and aerated with oxygen and mixtures of O₂ and CO₂ did not yield tyrothricin.

Negative results were likewise obtained with submerged flask cultures in which hydrolyzed casein, peptone, yeast extract, potato extract, gelatin, molasses, brain-heart infusion, milk whey or combinations of these, with and without glucose, were used in place of tryptone.

That tyrothricin is not formed under such conditions rather than being present in a bound form seems indicated by the fact that it cannot be recovered from the cellular material of such cultures by hydrolysis with acid, alkali or proteolytic enzymes, or by extraction with various organic solvents.

These experiments and numerous others indicate that the metabolism of *B. brevis* is modified in aerated submerged cultures containing complex nitrogenous nutrients so that tyrothricin is not formed, or at least does not accumulate.

EXPERIMENTS WITH SYNTHETIC MEDIA

While developing a synthetic medium which will permit abundant growth of *B. brevis* in stationary cultures, it was noted that nitrate-nitrogen is not utilized by the bacillus whereas ammonium-nitrogen permits scant growth; asparagin yields as much growth as tryptone or hydrolyzed casein. A mixture of growth factors (thiamin, pyridoxine, inositol, uracil, adenine, guanine and pantothenic, nicotinic, aspartic and p-aminobenzoic acids) stimulates growth in ammonium-nitrogen and asparagine media although these substances are not essential. The basal medium described below, with 0.25 per cent asparagine as nitrogen source, gave the best growth of *B. brevis* in stationary cultures.

Glucose.....	10.0 gm.
K ₂ HPO ₄	0.5 gm.
KH ₂ PO ₄	0.5 gm.
MgSO ₄ ·7H ₂ O.....	0.2 gm.
NaCl.....	0.01 gm.
MnSO ₄ ·4H ₂ O.....	0.01 gm.
FeSO ₄ ·7H ₂ O.....	0.01 gm.
CaH ₂ (PO ₄) ₂ ·H ₂ O.....	2 ml. of a saturated solution
Distilled water.....	998 ml.

Glucose was sterilized separately to avoid observed toxic effects due to caramelization.

Tyrothricin is readily formed in aerated submerged cultures of asparagine medium in contrast to its lack of formation in complex nitrogenous media. It can be isolated from such cultures as a fine, white powder in amounts ranging from 100 to 300 mg. per liter of medium. These yields are equivalent to those from stationary tryptone cultures.

Tyrothricin from submerged cultures is biologically and chemically similar to that obtained from shallow tryptone cultures. Ultra-violet absorption spectrum studies indicate that it may contain a greater percentage of gramicidin than the latter.

Tyrothricin is formed also with glycine and d-glutamic acid (table 1). Two-gallon fermenters containing 5 liters of medium were used. The amino acids were present in 0.5 per cent concentration as compared to 0.25 per cent asparagine. The glutamic acid medium was adjusted to pH 7 with concentrated NaOH; the

TABLE 1

Formation of tyrothricin in asparagine, glycine and d-glutamic acid media under various air pressures and rates of stirring

SOURCE OF NITROGEN	TYPE OF FERMENTATION VESSEL	IMPOSED AIR PRESSURE	RATE OF STIRRING	YIELD OF TYROTHRICIN
		<i>lbs. per sq. inch</i>	<i>r.p.m.</i>	<i>mg. per liter</i>
Asparagine	Glass-lined	0	60	120*
d-Glutamic acid	Glass-lined	0	0	145
d-Glutamic acid	Glass-lined	0	60	180
d-Glutamic acid	Glass-lined	5-8	60	162
d-Glutamic acid	Glass-lined	15	60	166
d-Glutamic acid	Iron	0	60	171
Glycine	Glass-lined	0	0	60
Glycine	Glass-lined	0	60	91

* This figure represents only part of the tyrothricin in the culture since an estimated 20 to 30 per cent was lost in the isolation process.

reaction of the remaining media was close to neutrality. The rate of air flow was 1.5 liters per minute.

The yields with glycine are significantly smaller than those with the other media: 60 and 91 mg. of tyrothricin per liter as compared to 145, 162, 166, 171 and 180 mg. per liter of glutamic acid medium and at least 120 mg. per liter of asparagine medium. Fifteen pounds air pressure was no better than atmospheric. The same is true for variation in rate of stirring from 0 to 60 r.p.m. Iron does not inhibit tyrothricin formation since the yield from the iron vessel with glutamic acid medium is equivalent to those from the glass-lined fermenters.

The following amino acids are also satisfactory sources of nitrogen for synthesis of tyrothricin in submerged cultures: dl-phenyl alanine, dl-aspartic acid, dl- α -alanine, l-hydroxyproline, dl-threonine, dl-serine, dl-valine, dl-leucine, l-proline, l-histidine monohydrochloride, dl-isoleucine, and also β -alanine. Negative or variable results have been obtained with l-cystine, dl-lysine, l-trypto-

phane and dl-methionine. The tests for tyrothricin in the amino acid media were only qualitative.

Figure 1 shows quantitative relations of glucose and glutamic acid utilization and synthesis of cell substance with respect to formation of tyrothricin. The conditions were: air flow, 2 liters per minute; air pressure, 50 lbs. per sq. in.; agitation, 60 r.p.m. Samples were withdrawn at intervals and centrifuged. The cellular material was assayed quantitatively for tyrothricin and for nitrogen (micro-Kjeldahl) as a measure of cell substance. The supernatant fluid was analyzed for glucose (Stiles, Peterson and Fred, 1927) and amino-nitrogen (Van Slyke), the latter values being used to calculate residual glutamic acid.

Growth was slow during the first 18 hours, followed by rapid development which was accompanied by rapid utilization of glutamic acid and by synthesis of

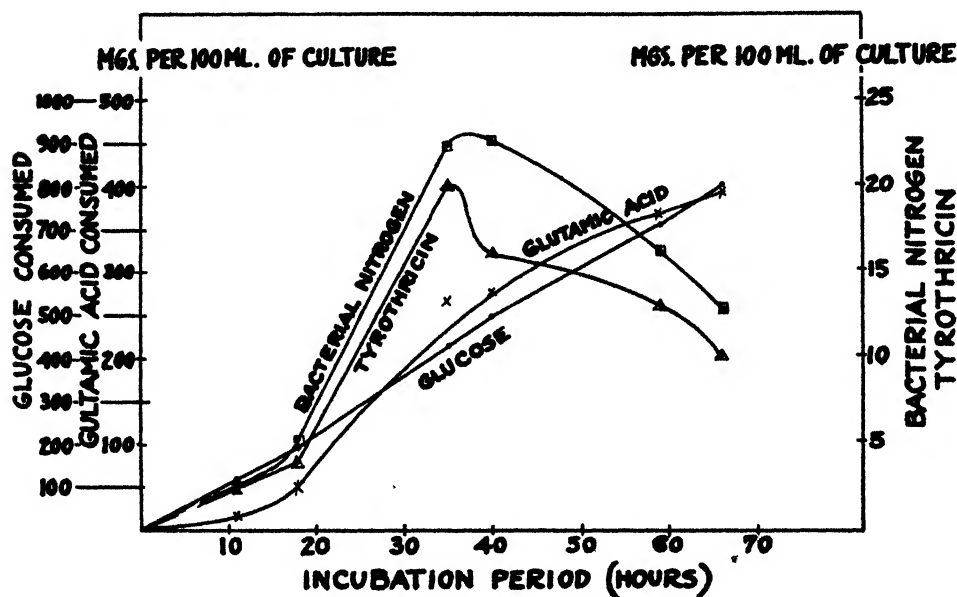


FIG. 1. UTILIZATION OF GLUCOSE AND GLUTAMIC ACID AND SYNTHESIS OF CELL SUBSTANCE BY *B. BREVIS* IN RELATION TO TYROTHRIN FORMATION

tyrothricin. Glucose was consumed at the same rate during all stages of growth. Maximum growth was reached in 35 to 40 hours after which there was a marked decrease in cell material which could be spun from the culture. This is due to autolysis which is reflected in an increasing opalescence of the supernatant fluid of centrifuged samples. The amount of tyrothricin is related to the quantity of cell material indicating that the bactericidal agent is closely associated with the latter. Furthermore, since formation of tyrothricin parallels the increase in bacterial substance during the logarithmic growth phase when little or no autolysis occurs, it appears that tyrothricin is an integral component of the viable bacterial cell and not merely a degradation product of autolysis.

The pH rises from 7.0 to a maximum of 8.4 which, in general, is the trend noted in stationary tryptone cultures.

Results of experiments with a mixture of 19 amino acids simulating hydrolyzed casein (Woolley and Hutchings, 1940) offer an explanation for the absence of tyrothricin in submerged cultures of complex nitrogenous media. Table 2 contains the results of two experiments on the influence of the amino-acid mixture on tyrothricin formation in the presence and absence of glutamic acid. Stationary and submerged cultures were compared. The former were grown in 125 ml. Erlenmeyer flasks containing 30 ml. of the glucose-mineral salts medium and the latter in 250 ml. Drechsel gas-washing bottles containing 125 ml. of the same medium. 0.25 per cent d-glutamic acid and amino acids equivalent to 0.2 per cent hydrolyzed casein were used.

In submerged cultures, the bactericidal agent is not formed when an amino-acid mixture is the sole source of nitrogen although there is good growth. More-

TABLE 2
Effect of amino acids on tyrothricin formation in submerged and stationary cultures

GLUCOSE-MINERAL SALTS PLUS ADDENDA AS BELOW	TURBIDITY*	ACTIVITY†				
		ml. of supernatant used				
		0.2	0.4	0.6	0.8	1.0
Submerged cultures						
Glutamic acid.....	14	+	+	+	+	+
Amino acid mixture.....	33	—	—	—	—	—
Glutamic acid plus amino acids.....	18	—	—	—	—	—
Stationary cultures						
Glutamic acid.....	18	+	+	+	+	+
Amino acid mixture	17	+	+	+	+	+
Glutamic acid plus amino acids....	17	+	+	+	+	+

* Readings on Evelyn photometer. Uninoculated medium = 92.

† Lysis of *M. conglomeratus*; + indicates complete lysis.

over, the amino acids *prevent* the formation of tyrothricin in the glutamic acid medium which itself is suitable for the synthesis of tyrothricin.³

In stationary cultures, however, tyrothricin is formed in both media, and the addition of the amino-acid mixture to the glutamate medium does not inhibit formation of the bactericidal agent. The amino-acid mixture changes the characteristics of the synthetic medium so that it behaves like complex nitrogenous media in which tyrothricin is formed only in shallow stationary cultures.

The effect of the amino acids in submerged cultures cannot be ascribed to any single amino acid or to certain groups of three or four.

The failure of *B. brevis* to synthesize tyrothricin in complex nitrogenous media may, therefore, be due to the amino acids of the latter. The active components of tyrothricin are polypeptides composed of approximately ten amino acids. It may be that one or more of the amino acids of the mixture (or nitrogen com-

³ Hydrolyzed casein will also inhibit tyrothricin formation in glutamic-acid medium under submerged conditions.

plexes) replaces characteristic amino acids in tyrothricin, resulting in the formation of tyrothricin-like polypeptides which do not have the biological or chemical properties of the bactericidal agent.

SUMMARY

Tyrothricin is synthesized by *Bacillus brevis* in shallow stationary cultures of complex nitrogenous media, but it is not formed, or at least does not accumulate, in aerated submerged cultures of such media.

However, tyrothricin is formed in both submerged and stationary cultures in a synthetic medium consisting of glucose, inorganic salts and asparagine. Glycocoll, d-glutamic acid and other amino acids can be substituted for asparagine.

The bactericidal agent is not produced in synthetic media under submerged conditions in the presence of a mixture of 19 amino acids. It is suggested that the failure of tyrothricin to appear in submerged cultures containing complex nitrogenous substances is associated with the amino acids of the latter.

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AN ANAEROBIC CULTURE TUBE FOR DETERMINING CO₂/H₂ RATIOS OF COLIFORM BACTERIA

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The significance of gas production by bacteria as a means for establishing natural species or groups of species was recognized by Theobald Smith (1890 and 1893). He showed that the bent tube, which had been used by chemists for years, was useful as a bacteriological culture tube which could also be used to measure gas production by bacteria. Although he was able to demonstrate differences in the kinds and amounts of gases produced by organisms with the use of this tube, he was aware of the inaccuracies in his quantitative analysis resulting from the absorption of the gases by the culture medium. His work indicated, however, that studies on the kinds and amounts of gases produced by bacteria would reveal relationships and differences not detectable by other means. Furthermore, he observed gas production from glucose to be one of the more permanent characteristics of the colon cultures he studied.

Rogers, Clark, and Davis (1914), using very accurate methods of gas analysis, found that colon organisms isolated from milk and milk products could be separated into two well-defined groups on the basis of CO₂/H₂ ratio. In a low ratio group the CO₂/H₂ value was approximately 1.0, and in a high ratio group the ratio ranged from 1.5 to 2.8. The ratio value and volume of gas produced by a given culture were found to be quite constant, and correlated closely with the fermentation of carbohydrates and alcohols, indicating that the gas ratio denoted lines of natural relationship within the colon-aerogenes group.

Having found the gas ratio to be a promising means for the characterization of members of the colon-aerogenes group, Rogers, Clark, and Evans (1914) isolated organisms of this type from bovine feces in an effort to establish the habitat of the two ratio groups. Of a total of 150 cultures, 149 had a CO₂/H₂ ratio of 0.98–1.20 and one gave a high ratio value. These results indicated that the predominant colon type in bovine feces was the low ratio group.

The question of whether the presence of colon-like organisms on grains indicated fecal pollution led Rogers, Clark, and Evans (1915) to study 166 strains of this type occurring naturally on grains. Seven of the cultures produced only carbon-dioxide, eight gave a ratio of 1.06 but differed from the *Escherichia coli* group in other respects, and the remaining 151 cultures gave a ratio of 1.90–3.00. By means of the gas ratio and other tests, they concluded that none of the types found on grains agreed with the characteristic flora of bovine feces.

In a study of bacteria of the colon-type occurring in human intestines, Rogers, Clark, and Lubs (1918) studied 177 cultures in an effort to establish the charac-

¹ Now a Lieutenant in the Sanitary Corps of the United States Army.

teristics of these organisms from this source. Of the 177 cultures, 131 gave CO_2/H_2 ratios of approximately 1.06, while the remaining varied from 1.5–2.7. This again indicated that the low ratio group was of intestinal or fecal origin.

The results of the investigations of Rogers and his associates showed that members of the genus *Escherichia* were included in the low ratio group and were found predominantly in feces or sources contaminated with fecal material. The high ratio group included *Aerobacter* organisms which were most frequently found on grains or were primarily of vegetable origin.

Speck and Stark (1942), using methods similar to those of Rogers and his associates for determining gas ratios, confirmed the ratio values established by those workers for the *Escherichia* and *Aerobacter* genera. In addition, they found that the CO_2/H_2 ratio presented promising means for characterising species in the genus *Proteus*.

Many tests have been introduced which permit conclusive recognition and separation of *Escherichia* and *Aerobacter* organisms. Yet cultures are often encountered possessing properties of both genera which make their identity questionable. Although Rogers and his associates clearly demonstrated the excellent reliability of the gas ratio in separating colon-aerogenes organisms, no practical use has been made of this property for such purposes. This mainly has been caused by the special culture and gas analysis equipment, and the time, which are required to make accurate CO_2 and H_2 determinations. In view of the significance of CO_2/H_2 ratios in separating *Escherichia* and *Aerobacter* organisms and in recognizing species of the genus *Proteus*, an anaerobic culture tube was devised in which the CO_2/H_2 ratio of these organisms could be determined directly and with no additional apparatus required, other than a leveling bulb and tube.

EXPERIMENTAL

The only measurable gases produced by the coliform bacteria in the dissimilation of glucose are carbon-dioxide and hydrogen. It remained then only to determine the total volume of gas and the volume of one of these constituent gases, and the other could be determined by difference. As the carbon-dioxide could be absorbed with alkali, the method selected was to determine the total gas volume and carbon-dioxide produced. The gas remaining after absorbing the CO_2 was measured as hydrogen.

The anaerobic fermentation tube designed for these experiments is essentially a bent fermentation tube to which is attached a stopcock (fig. 1).² Also a bulb was placed in the collection arm to aid in breaking bubbles formed when the tube and medium were evacuated, and so prevent the medium from foaming into the other arm of the tube. Except for a rubber stopper the tube is made entirely of Pyrex glass. The long arm of the tube is 11 mm. tubing, with a 22 mm. bulb blown in it 20 cm. from the top. The short arm is made by bending the 11 mm. tubing to a 10° angle with the long arm. This is extended 3 cm. beyond the

² We wish to thank the Fisher Scientific Co., Pittsburgh, for supplying a number of these tubes for experimental work.

bend of the elbow and then a 6 cm. length of 8 mm. tubing sealed on to it. A 2 mm. bore stopcock is sealed onto the 8 mm. tubing 3.5 cm. down and 15 mm. to the side of the short arm. The other arm of the stopcock is extended 3.5 cm. for connections and adding of reagents. The mouth of the short arm of the tube is closed with a OOO rubber stopper. Using the specified inside diameter tubing for the collection arm one cm. length corresponds to approximately 1 ml. volume.

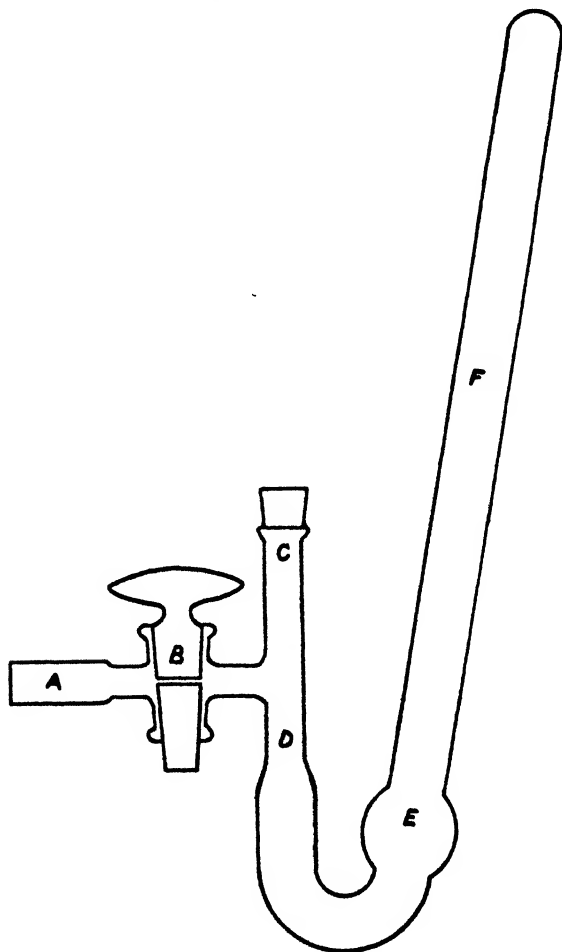


FIG. 1. ANAEROBIC CULTURE TUBE FOR DETERMINING CO₂/H₂ RATIOS OF COLIFORM BACTERIA

For more exact determinations of volumes the collection arm can be calibrated. This, however, is unimportant when the main purpose is to determine ratio values.

The medium employed contained 1 per cent Bacto proteose-peptone, 1 per cent glucose, and 0.5 per cent K₂HPO₄ (dry) dissolved in distilled water (RPP medium). In the tests where Rogers' medium (R) was used, it was prepared by dissolving 1 per cent Witte peptone and 0.5 per cent K₂HPO₄ (dry) in dis-

tilled water. This was steamed for 20 minutes, the precipitate filtered off, the solution made up to volume, and then 1 per cent glucose was added. Five ml. were placed in the anaerobic tube, sterilized at 120°C. for 15 minutes. The culture to be tested was grown for at least one 24-hour period at 30°C. in the medium of the same composition in which it was to be tested for the gas ratio, and 0.2 ml. of the culture inoculated into the anaerobic tube. An alternate procedure was to place 7 ml. of medium in a plugged test tube, sterilize and inoculate as described for the anaerobic tube, and then place 5 ml. of the inoculated medium pipetted aseptically into the sterile anaerobic tube. Most of the gas ratios were made by sterilizing only the peptone and buffer in the anaerobic tube and then adding aseptically 1 ml. of 5 per cent glucose sterilized by autoclaving. Four cultures were tested later in the study to determine if sterilization of the glucose with the other constituents of the medium had any effect on the gas ratio. The results showed no significant differences in the ratios when media prepared by either method was used.

The tube should be cleaned occasionally with acid cleaning solution. The stopcock (*B*) is lubricated with cello-grease which resists the autoclaving temperature more satisfactorily than lighter greases. The opening (*A*) is plugged with cotton and the stopcock (*B*) turned to the open position. The culture medium is measured into the tube through the opening (*C*) which is then closed with a rubber stopper. The tube is sterilized by autoclaving at 120°C. for 15 minutes in an inverted position with all the medium in the arm (*F*). After sterilization and cooling, the tube is inoculated through (*C*). The rubber stopper is then firmly seated and the rubber-glass seal reinforced with plasticene. The rubber stopper should be inspected frequently to be certain that it seats firmly in the tube.

To obtain anaerobic conditions, the stopcock (*B*) is turned to the closed position and the cotton plug removed from (*A*) which is then attached to a vacuum pump with pressure tubing. A good water aspirator can usually produce a satisfactory vacuum, although the efficiency of the pump should be ascertained. The tube is inverted with the medium in the arm (*F*) and the air is released by opening the stopcock. The bulb (*E*) tends to keep the medium from being carried over by boiling, but this should be supplemented by controlling the rate of evacuation with the stopcock. A satisfactory vacuum is present after the medium has boiled for 1-2 minutes. The stopcock is then closed and the tube is incubated in any position for 24 hours at 30°C. A limited number of experiments indicated that 30°C. was the optimum temperature for obtaining maximum gas volumes and characteristic CO_2/H_2 ratios of coliform organisms.

Determination of the ratio of CO_2 to H_2 in the tube after incubation is a relatively simple procedure and requires only about 5-10 minutes. To aid in releasing the CO_2 dissolved or combined in the medium, one ml. of 5 per cent HCl is added to (*A*) and cautiously allowed to enter through the stopcock (*B*). Any air trapped below the surface of the acid in (*A*) can be removed with a capillary pipette. The acidified medium is allowed to stand for 2-3 minutes. The total amount of gas present is then determined. (*A*) is filled with freshly

boiled and cooled distilled water. A leveling bulb and rubber tubing leading from it are filled with water and the other end of the tubing connected to (A). Care must be taken that the rubber tube is free from air and that no air is trapped when the tube is connected with (A). The water is allowed to enter slowly through the stopcock, until the vacuum is dissipated. All the gas is collected ahead of the incoming water in the arm (F) and the total volume after leveling is measured. Care should be taken to prevent gas from remaining in (D), as some CO₂ in the tube will be absorbed by the medium if mixed with it after dissipating the vacuum.

The relative volumes of CO₂ and H₂ are obtained by absorbing the CO₂ with NaOH and measuring the remaining gas as H₂. With the stopcock open, the leveling bulb is lowered until about 2 ml. of liquid are withdrawn from the tube. The stopcock is then closed and the liquid shaken from (A). About 2 ml. of 50 per cent NaOH is added to (A) and allowed to enter through the stopcock. With all the gas in the arm (F) no absorption will take place until all the NaOH has entered the tube, the stopcock has been closed, and the alkali mixed by inversion. The tube should be inverted several times and allowed to stand for 1-2 minutes to insure complete absorption of all the CO₂.

The gas remaining in the tube is measured by again attaching the leveling bulb and proceeding as for the total gas volume. The remaining gas is measured as hydrogen. Uninoculated control tubes have shown that the volume of residual gas after evacuation is negligible when a satisfactory vacuum pump is used for evacuating the tube before incubation. The gas ratio (CO₂/H₂) can then be calculated by the following formula:

$$\text{CO}_2/\text{H}_2 = \frac{\text{total gas volume} - \text{hydrogen volume}}{\text{hydrogen volume}}$$

Experimental data show that CO₂/H₂ ratios of *E. coli* and *A. aerogenes* determined by this method are in good agreement with those found by other workers using more elaborate equipment and time consuming methods.

RESULTS

Medium. Rogers and his co-workers and Speck and Stark (1942) have used Witte peptone as the nitrogenous component of the nutrient broth in their gas ratio studies. The latter have indicated that it is possible for coliform organisms to produce gas from the peptone, resulting in an alteration of the gas ratio, and emphasize the importance of selecting a medium in which the gases produced represent as nearly as possible only those formed in the metabolism of glucose. In the present investigation it seemed advisable to substitute a more readily obtainable peptone than Witte in the nutrient broth.

Bacto proteose-peptone, which is recommended for use in the methyl-red and Voges-Proskauer tests was found to be satisfactory. A number of tests in which coliform organisms were inoculated into the fermentation tube containing 1 per cent Bacto proteose-peptone and 0.5 per cent of K₂HPO₄, indicated that no gas was produced from this peptone. The CO₂/H₂ ratios obtained from five

Escherichia and *Aerobacter* cultures grown in Rogers' (R) medium (Witte peptone 1 per cent, glucose 1 per cent, K_2HPO_4 0.5 per cent) and in a medium (RPP) of the same composition, but containing Bacto-proteose-peptone instead of Witte, were found to agree very closely (table 1). The latter medium (RPP) was used, therefore, in all subsequent gas ratio determinations.

Period of incubation. Gas ratio studies in the coliform group, heretofore, have been used mainly to indicate the relative amounts of CO_2 and H_2 remaining after the culture has ceased growing, and seven days at $30^\circ C$. has generally been allowed for this activity. It was felt that the gas ratio would be of much more practical value if the differences in relative amounts of CO_2 and H_2 could be demonstrated in a shorter incubation period. Six cultures representing the four *Escherichia* and *Aerobacter* species were each incubated for one, two, and seven days at $30^\circ C$. in RPP medium and the gas ratio determined for each incubation period (table 2). The total volume of gases and the CO_2/H_2 ratios were essen-

TABLE 1
The effect of medium on the CO_2/H_2 ratio

CULTURE	MEDIUM	TOTAL GAS	H_2	CO_2	CO_2/H_2
		ml.	ml.	ml.	
<i>E. coli</i> (Co 9)*	R	5.3	2.6	2.7	1.04
<i>E. coli</i> (Co 9)*	R-PP	6.4	3.0	3.4	1.13
<i>A. aerogenes</i> (3)*	R	15.8	5.5	10.3	1.87
<i>A. aerogenes</i> (3)*	R-PP	14.9	5.0	9.9	1.98
<i>A. aerogenes</i> (AA165)	R	14.8	4.8	10.0	2.08
<i>A. aerogenes</i> (AA165)	R-PP	14.8	4.7	10.1	2.15
<i>A. cloacae</i> (65)	R	11.7	3.3	8.4	2.54
<i>A. cloacae</i> (65)	R-PP	16.7	6.1	10.6	1.90
<i>E. freundii</i> (14)	R	7.6	3.8	3.8	1.00
<i>E. freundii</i> (14)	R-PP	7.8	3.8	4.0	1.05

* Incubated 7 days at $30^\circ C$.; all others incubated 2 days at $30^\circ C$.

tially the same after 24 hours as after 7 days, indicating that most of the gas production was accomplished in the first 24 hours. These results indicated that the gas ratios could be determined satisfactorily after one day at $30^\circ C$., and this period of incubation was used in the remainder of the analyses unless otherwise indicated.

Ratios of typical Escherichia and Aerobacter cultures. Eight cultures of *E. coli* and seven cultures of *Escherichia freundii* were tested for the CO_2/H_2 ratio produced in glucose broth (table 3). Some of the cultures had been recently isolated while others had been carried on laboratory media for varying numbers of years. The cultures of *E. coli* and *E. freundii* had been isolated from human feces, water, and milk. There were no correlations in ratio values or gas volumes with source or age of the culture. The small total volume of gas and the CO_2/H_2 ratio of approximately 1.0 given by these cultures are characteristic of those which have been established for *Escherichia* organisms grown under similar conditions. With

E. freundii, a citrate-utilizing organism, belonging to the genus *Escherichia* the usefulness of the citrate test for differentiation of *Escherichia* and *Aerobacter* genera becomes limited. This is in accord with Stark and Straughn's views (1941).

Fourteen cultures of *Aerobacter aerogenes* and *Aerobacter cloacae* were likewise studied. Both species gave a relatively larger total volume of gas and a CO₂/H₂ ratio of approximately 2/1, which are characteristic for *Aerobacter* organisms. Although there was a considerable difference in the time which the cultures had been carried on laboratory media, each culture gave a CO₂/H₂ ratio characteristic of the genus. The ratio values of *Aerobacter* cultures do not, however,

TABLE 2

The effect of the period of incubation on the CO₂/H₂ ratio produced in RPP medium

CULTURE	TIME					
	1 day		2 days		7 days	
	Total gas	CO ₂ /H ₂	Total gas	CO ₂ /H ₂	Total gas	CO ₂ /H ₂
	ml.		ml.		ml.	
<i>A. aerogenes</i> (3).....	14.3	1.92	14.8	2.02	15.2	1.92
<i>E. coli</i> (Co 9).....	7.0	1.03	7.9	1.14	6.4	1.13
<i>E. coli</i> (5).....	7.5	.93	7.3	1.14	7.0	1.12
<i>A. aerogenes</i> (AA165).....	14.1	2.13	13.9	2.23	15.1	2.15
<i>A. cloacae</i> (65).....	16.9	1.77	16.7	1.90	16.3	2.02
<i>E. freundii</i> (14).....	7.2	1.00	7.8	1.05		

TABLE 3

Summary of CO₂/H₂ ratios and total gas volumes of coliform cultures

CULTURE	NUMBER CULTURES	TOTAL GAS		CO ₂ /H ₂	
		Range	Average	Range	Average
		ml.	ml.		
<i>E. coli</i>	8	5.7-10.2	6.9	0.80-1.04	0.96
<i>E. freundii</i>	7	6.1- 9.5	7.7	0.88-1.03	0.97
<i>A. aerogenes</i>	3	14.1-15.7	14.7	1.92-2.13	2.02
<i>A. cloacae</i>	11	11.9-16.9	13.3	1.77-2.22	1.96
"Intermediates".....	3	5.8-10.1	7.6	0.89-1.07	0.98

normally fall in as narrow a range as do those of the genus *Escherichia*, but the limits of the high and low ratio groups do not overlap. There was no significant difference in the ratio of *A. aerogenes* and *A. cloacae* cultures.

Ratios of Coliform "intermediates". Three cultures of coliform bacteria were encountered, two of which were IMVIC— — — +, and the other + — — —. Only the former two produced H₂S. All of these cultures gave gas volumes and ratios characteristic of the genus *Escherichia*.

DISCUSSION

The anerobic gas tube described has natural limitations regarding the accuracy with which measurements of the actual volumes of CO₂ and H₂ can be made. For

instance, the CO_2 dissolved or combined in the medium may be influenced by different residual pressure in the tube after the growth of the culture. Also, depending upon the efficiency of the vacuum pump, a very small amount of unevacuated air may remain in the tube and be measured in the volumes of total gas and hydrogen. But for purposes for which this tube was designed, these factors can be entirely disregarded, as evidenced by the agreement of the CO_2/H_2 values obtained by this tube with those determined by more precise methods.

The CO_2/H_2 ratios of the *Escherichia* and *Aerobacter* organisms, representing two different types of glucose metabolism, are more fundamental than many of the characteristics now used to describe these genera. Although the gas ratio has not been employed as widely in studies of the coliform organism as have more convenient tests, its reliability has so far not been questioned. This should, however, be more completely ascertained by more extensive studies of the gas ratio. The CO_2/H_2 value may show particular usefulness in determining relationships among the coliform "intermediates." The few "intermediates" employed in this study gave CO_2/H_2 ratios characteristic of the genus *Escherichia*.

The advantages of this tube over other methods of determining CO_2/H_2 ratios are its simplicity of design, relative low cost and facility and reliability with which it can be used by persons not familiar with gas analysis apparatus. Using the method described, all cultures which have been tested so far have produced their characteristic CO_2/H_2 ratio in 20-24 hours. This incubation period is sufficiently short to permit the use of this tube in routine differentiation studies.

SUMMARY

An anaerobic culture tube was designed in which could be measured the relative volumes of CO_2 and H_2 produced by coliform bacteria from glucose. The results of a number of experiments indicated that the characteristic CO_2/H_2 ratio of *Escherichia* and *Aerobacter* organisms can be ascertained after a one-day incubation period at 30°C .

The ratio values of coliform bacteria obtained with the anaerobic culture tube are in good agreement with those reported by other workers using customary gas analysis equipment. The facility with which the tube can be used and the reliability of the data obtained with it indicate that it could be particularly useful in routine identification of members of the *Aerobacter* and *Escherichia* genera when such separation is desired.

The anaerobic fermentation tube may be of value in studying the relationship of the coliform "intermediates" to the *Escherichia* and *Aerobacter* genera.

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THE B-VITAMIN REQUIREMENTS OF THE PROPIONIBACTERIA

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The nutritional requirements of the Propionibacteria have long been regarded as complex. It has been shown that amino acids, although beneficial, are not essential (Fromageot and Loroux, 1936; Tatum, Wood and Peterson, 1936a; Wood, Anderson and Werkman, 1938). They may be replaced by $(\text{NH}_4)_2\text{SO}_4$, but growth of many species is seriously impaired upon such a medium. Thiamin has been reported by Tatum, Wood and Peterson (1936b) as stimulatory to certain species. However, Silverman and Werkman (1939) found that cultures could be "trained" to grow vigorously in its absence. Riboflavin has been reported by Wood, Anderson and Werkman (1938) as stimulatory, but it can apparently be dispensed with (Krauskopf, Snell and Peterson, 1939). Pantothenic acid has been shown by Krauskopf, Snell and Peterson (1939) to be required by several species. No success in the culturing of any of these organisms on a completely synthetic medium has been reported. Perhaps the nearest approach was that of Wood, Tatum and Peterson (1937), who succeeded in growing a number of species in a synthetic $(\text{NH}_4)_2\text{SO}_4$ medium supplemented with an ether extract of an aqueous yeast extract. Growth was rather meager, however, and addition of hydrolyzed casein was necessary for sub-culturing.

It was the purpose of this investigation to ascertain which, if any, of the Propionibacteria could be grown on an essentially synthetic medium containing all of the known B-vitamins; and, if growth were obtained, to show which of the vitamins were essential for growth. The organisms investigated were the following: *Propionibacterium jensenii* (1), *P. jensenii* (29), *P. pentosaceum* (4), *P. thonii* (15), *P. rubrum* (19), *P. peterssonii* (20), and *P. technicum* (22), all derived from Van Niel's strains (1928); and *P. arabinosum*, and *P. zeae*, derived from Hitchner's (1932) original strains.

EXPERIMENTAL

Medium

The basal medium used throughout the investigation had the following composition:

Glucose.....	10.0 g.
Acid hydrolyzed, charcoal treated, vitamin-free casein.....	5.0 g.
$\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$	8.0 g.
Cystine.....	0.1 g.
Tryptophane.....	0.025 g.
Adenine.....	0.01 g.
Guanine.....	0.01 g.

Uracil.....	0.01 g.
Xanthin.....	0.01 g.
K ₂ HPO ₄	0.25 g.
KH ₂ PO ₄	0.25 g.
MgSO ₄ ·7H ₂ O.....	0.10 g.
NaCl.....	0.005 g.
FeSO ₄	0.005 g.
MnSO ₄ ·4H ₂ O.....	0.005 g.
Water to make.....	one liter
pH 6.0	

The casein hydrolysate was prepared by refluxing 100 g. of Labco "Vitamin-Free" casein with one liter of constant boiling HCl for 8-10 hours. The HCl was then removed by repeated vacuum distillation, and the pH adjusted to 3.0. 10 g. of Darco G-60 decolorizing charcoal were then added and the mixture shaken for fifteen minutes. The charcoal was then removed by filtration and the solution preserved under toluene.

The vitamins investigated and the amounts in which they were used are as follows:

Nicotinic acid.....	1.0 γ/culture
Pantothenic acid.....	1.0 γ/culture
Riboflavin.....	1.0 γ/culture
Thiamin.....	1.0 γ/culture
Pyridoxin.....	1.0 γ/culture
Inositol.....	1.0 γ/culture
p-Aminobenzoic acid.....	1.0 γ/culture
Biotin.....	0.1 γ/culture
Folic acid.....	1.0 mg. unit (described below)/culture

These amounts were thought to be sufficient in the light of the requirements of other organisms. All of the vitamins employed were crystalline products with the exception of folic acid, which was a concentrate of potency 3,000. The amount of folic acid added per culture was equivalent to one milligram of Liver Fraction B (Wilson).

Procedure

Inoculum tubes containing 10 ml. of the basal medium plus 1 mg. of Difco yeast extract were inoculated from yeast-extract glucose stabs and incubated forty-eight hours at 33°C. The cells were centrifuged out, the supernatant medium decanted, sterile saline added, and the cells suspended by shaking. They were again centrifuged out and resuspended in sterile saline. It was hoped by this washing process to reduce to a minimum any carryover from the yeast-extract inoculum medium. The suspensions of the various strains were diluted to an approximately uniform, slight turbidity, and 1 ml. of this suspension added to 150 ml. of double-strength, sterile, basal medium. A 5 ml. amount of this inoculated medium was added to each of a series of tubes containing all of the B-vitamins but one, and also control tubes containing all of the vitamins,

none of the vitamins, 1 mg. yeast extract, and 10 mg. yeast extract—all of these dissolved in 5 ml. of water, so that the final culture had a volume of 10 ml. By thus inoculating the medium before distribution to the various tubes it was hoped to obtain uniform inoculation, personal experience and the work of Chaix and Fromageot (1935) having indicated that the growth of the *Propionibacteria* is very sensitive to the size of the inoculum. All of the above operations were carried out aseptically. The cultures were incubated for three and one-half days at 33°C. Growth was then estimated turbidimetrically with the aid of the thermoelectric turbidimeter described by Williams, McAlister and Roehm (1929).

RESULTS

The results of the preliminary experiment described above are shown in table 1. The amount of growth is indicated by the galvanometer reading, a reading of zero corresponding to pure water and a reading of 100 indicating complete opacity. It must be borne in mind that the galvanometer response is not linear. Thus the difference between a reading of 80 and 90 indicates a considerably greater increment of growth than the difference between 10 and 20.

These results would seem to indicate that *P. pentosaceum*, *P. arabinosum*, *P. zeae*, and probably *P. rubrum*, require factors other than the eight B-vitamins tested. The remaining strains grow optimally, or nearly so, in the presence of these eight B-vitamins. The individual vitamins required by each organism are also indicated. Confirmation of these indications will be considered separately for each strain studied.

P. jensenii-1: The data in table 1 indicate that this organism requires only pantothenic acid and biotin. This was confirmed by culturing the organism through seven serial transfers on the basal medium supplemented by pantothenic acid and biotin. Transfers (in this and the following cases, unless otherwise indicated) were made at three-day intervals, one drop of the old culture serving as inoculum. Vigorous growth was maintained throughout the subculturing.

P. jensenii-29: Pantothenic acid and biotin are indicated in table 1 as the required factors. The organism was carried through seven serial transfers on the basal medium supplemented by these two vitamins; however, growth was very poor in the sixth and seventh tubes. Much better growth was obtained toward the end of the subculture series by addition of *p*-aminobenzoic acid. Even with this addition, however, growth was sub-optimal.

P. pentosaceum-4: This organism grew only slightly on the basal medium plus all eight vitamins. This slight growth, however, was carried through four serial transfers (on the basal medium plus eight vitamins) and, although still weak, there was more growth in the final subculture than in the original culture. Growth on this medium was slow, six days being allowed between subcultures. Subcultures could not be maintained on the basal medium plus pantothenic acid alone.

P. thonii-15: Pantothenic acid, biotin, and possibly thiamin and/or *p*-aminobenzoic acid are indicated by table 1 as probably required factors for this or-

ganism. It was carried through seven serial transfers on the basal medium plus these four vitamins, abundant growth occurring in all subcultures. With only pantothenic acid and biotin added, however, growth failed on the fourth subculture. Addition of either thiamin or *p*-aminobenzoic acid in addition to pantothenic acid and biotin allowed growth to continue through seven transfers, although growth was somewhat less abundant than when all four vitamins were present.

P. rubrum-19: This organism in the preliminary experiments grew slightly on the completely vitamin-supplemented medium (table 1), but all succeeding attempts to secure growth on this medium were unsuccessful.

TABLE 1

	<i>P. jensenii</i> -1	<i>P. jensenii</i> -29	<i>P. pentosaceum</i> -4	<i>P. thomii</i> -15	<i>P. rubrum</i> -19	<i>P. peterssonii</i> -20	<i>P. technicum</i> -22	<i>P. arabinosum</i>	<i>P. zeae</i>
None.....	7	51	15	6	4	17	6	3	4
1 mg. yeast extract.....	89	85	41	89	84	75	85	59	42
10 mg. yeast extract.....	97	92	99	94	95	91	91	99	96
All vitamins.....	87	79	17	84	28	65	90	4	4
All vitamins except nicotinic acid.....	84	82	*	84	23	73	90	*	*
All vitamins except pantothenic acid.....	7	20	*	7	4	15	9	*	*
All vitamins except riboflavin.....	87	80	*	86	29	72	89	*	*
All vitamins except thiamin..	81	75	*	71	32	67	86	*	*
All vitamins except pyridoxin	87	81	*	85	4	73	91	*	*
All vitamins except inositol..	87	81	*	84	49	72	87	*	*
All vitamins except <i>p</i> -aminobenzoic acid.....	84	71	*	77	30	74	88	*	*
All vitamins except biotin...	28	49	*	17	7	37	87	*	*
All vitamins except folic acid.	84	80	*	85	43	71	91	*	*

* Growth (observed visually) substantially the same as blank with no added vitamins.

P. peterssonii-20: Pantothenic acid and biotin were indicated as the essential factors for this organism. On the basal medium supplemented by these two vitamins, growth failed on the fifth subculture. Growth through seven transfers was permitted by addition of thiamin and *p*-aminobenzoic acid.

P. technicum-22: According to the data of table 1 this organism should require only pantothenic acid. This was confirmed by culturing the organism through seven serial transfers on the basal medium supplemented by pantothenic acid only. Vigorous growth was maintained on this medium.

P. arabinosum: This organism grew only very slightly on the completely vitamin-supplemented medium in the preliminary experiments (table 1). It was found, however, to be subculturable on this complete medium. It was carried through five serial transfers and, as in the case of *P. pentosaceum*, growth

became increasingly abundant with succeeding subcultures. Subcultures could not be maintained with pantothenic acid only as a supplement.

P. zeae: This organism showed no growth on the basal medium supplemented with the eight vitamins.

SUMMARY AND DISCUSSION

Five of the nine *Propionibacteria* studied in this investigation were found to grow satisfactorily through repeated subculture on a medium, synthetic with the exception of a charcoal-treated "vitamin-free" casein hydrolysate. All of these five required pantothenic acid. This confirms the earlier work of Krauskopf, Snell, and McCoy (1939). Four of the five required biotin. It seems probable that the ether extract of yeast extract used by Wood, Tatum and Peterson (1937) in the culture of *Propionibacteria* was effective due to its content of pantothenic acid and biotin. Nicotinic acid, riboflavin, pyridoxin, inositol, and folic acid were, under the conditions of this experiment, without stimulatory effect. The role of thiamin and of *p*-aminobenzoic acid is somewhat obscure. For certain of the organisms one or the other or perhaps both are apparently essential for continued subculture. Silverman and Werkman (1939) found that the thiamin requirements of *Propionibacterium pentosaceum* were widely variable depending upon the previous history of the culture. Response to *p*-aminobenzoic acid by *P. thonii* has been observed in the course of this investigation to be quite irregular. On some occasions this organism has shown practically no response (as in table 1) and at other times, under apparently identical conditions, growth would be more than doubled by addition of *p*-aminobenzoic acid. These facts, together with the apparent adaptation of *P. pentosaceum* and *P. arabinosum* to slow growth in an unfavorable medium, appear to support the conclusion reached by Wood, Anderson and Werkman (1938) in reviewing the then known nutritional requirements of the *Propionibacteria*, namely, that these organisms are endowed with rather remarkable adaptive capacities.

P. pentosaceum, *P. rubrum*, *P. zeae*, and *P. arabinosum* apparently require a factor or factors in addition to the eight B-vitamins considered in this study. This factor (or factors) is present in yeast extract. It should also be noted (table 1) that with the exception of *P. technicum*, all strains are stimulated by yeast extract, over and above the stimulation of the eight B-vitamins. The nature of this stimulation is being investigated.

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NOTES

FURTHER STUDIES ON THE EIJKMAN REACTIONS OF SHIGELLA CULTURES

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Stuart *et al.* (1942) found that 15 cultures of *Shigella paradysenteriae*, with one exception, failed to grow while 17 *S. sonnei*, 4 *S. alkalescens* and one *S. dispar* (madampensis) cultures grew readily and fermented glucose at 45.5°C. Wood *et al.* (1943) found that the ability of *Shigella* species to reduce trimethylamine oxide corresponded to their Eijkman reactions. In view of this correlation it seemed advisable to extend the work on the Eijkman reactions of *Shigella*.

In the present work on 276 cultures a temperature of 45°C., $\pm 0.1^\circ\text{C}$., was found more satisfactory than 45.5°C. Inoculations were made from 24-hour broth cultures. Two loopfuls of group I and one loopful of group II species

TABLE 1

GROUP	SHIGELLA SPECIES	CULTURES TESTED	EIJKMAN REACTIONS			
			No growth	Growth	Slight acid	Strong acid
I	<i>Sh. dysenteriae</i>	6	6			
	<i>Sh. paradysenteriae</i>	61	61			
	<i>Sh. ambigua</i>	3	3			
	<i>Sh. sp.</i> (Newcastle type)	15	15			
	<i>Sh. equirulis</i>	1	1			
Totals.....		86	86	0	0	0
II	<i>Sh. sonnei</i>	17		1	2	14
	<i>Sh. alkalescens</i>	142		2		140
	<i>Sh. dispar</i> (madampensis)	22				22
	<i>Sh. ceylonensis</i>	9				9
Totals.....		190	0	3	2	185

(table 1) were inoculated into Difco Eijkman medium base with glucose. All cultures were incubated for 24 hours. Table 1 shows that none of 86 group I cultures grew at 45°C. After 24 hours at 45°C. the cultures were placed at 37°C. Seventy-nine group I cultures, after showing no visible growth for from 12 to 36 hours at 37°C., produced acid while 7 cultures failed to grow. In the previous work one carefully checked *S. paradysenteriae* gave a strong acid reaction at 45.5°C. Unfortunately this culture was not available for the trimethylamine test. All 190 group II cultures tested in the present work grew and 185 or 97.4 per cent produced strong acid from glucose at 45°C.

As pointed out by Neter (1942) *S. alkalescens* is not infrequently mistaken for *S. paradysenteriae*. To a lesser extent a similar condition holds true for *S. sonnei* cultures fermenting lactose much more slowly than the average strain. Diagnostic laboratories without adequate antisera could use either the Eijkman or the trimethylamine test or both to good advantage with *Shigella* cultures. Considering the large number of cultures tested the single group I, Eijkman positive exception and the single group I, trimethylamine positive exception (Wood *et al.* 1943) detract but little from the practicability of these two tests.

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A PRIMARY DIVISION OF THE GENUS SHIGELLA BASED ON THE TRIMETHYLAMINE TEST

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A recent survey (Wood and Baird 1943) of the Enterobacteriaceae has revealed that most of the species of this family are able to reduce trimethylamine oxide to trimethylamine. The only exceptions were noted in *Shigella* and *Erwinia*.

TABLE 1

GROUP	SHIGELLA SPECIES	NUMBER OF CULTURES	TRIMETHYLAMINE PRODUCTION	
			Neg.	Pos.
I	<i>S. dysenteriae</i>	16	16	
	<i>S. paradysenteriae</i>	87	86	1
	<i>S. ambigua</i>	7	7	
	<i>S. schmitzii</i>	1	1	
	<i>S. sp.</i> (Newcastle type)	5	5	
	<i>S. equirulis</i>	1	1	
Totals.....		117	116	1
II	<i>S. sonnei</i>	22		22
	<i>S. alkalescens</i>	98		98
	<i>S. madampensis</i> (dispar)	19		19
	<i>S. ceylonensis</i>	2		2
Totals.....		141	0	141

The work with *Shigella* has been extended to additional species and to a larger number of cultures of those species already examined in the hope that the findings might have some taxonomic value.

The results presented in Table I were obtained using a procedure already described (Wood and Baird 1943). They have been confirmed by repetition at three different intervals over a period of three months.

The division into two groups, one positive and the other (except for one *S. paradysenteriae* culture) negative for trimethylamine, is in striking agreement with the results of Stuart and Rustigian (1943) based on the Eijkman reactions. A primary separation of the species of this genus based on the trimethylamine test or on the Eijkman reactions appears to offer some advantage over the present one using mannitol fermentation (Bergey 1939, Glynn and Starkey 1939). As pointed out by Stuart and Rustigian (1943) the Eijkman reactions or the trimethylamine test or a combination of both may be of value to laboratories not equipped with antisera required for differential purposes.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

JOINT MEETING OF THE NEW JERSEY, THE EASTERN PENNSYLVANIA, AND
THE NEW YORK CITY BRANCHES

PRINCETON, N. J., MAY 15, 1943

NOTE ON ANTIBIOTIC SUBSTANCES ELABORATED BY AN *ASPERGILLUS FLAVUS* STRAIN AND BY AN UNCLASSIFIED MOLD. *Arthur E. O. Menzel, O. Wintersteiner and Geoffrey Rake*, The Squibb Institute for Medical Research, New Brunswick, N. J.

Aspergillic acid, the antibiotic substance elaborated by a strain of *Aspergillus flavus* (isolated and supplied by Dr. E. C. White) has been prepared by a simple extraction procedure. Preparations from plain tryptone media melted between 80° and 90°C., while preparations obtained from media containing brown sugar invariably melted above 100°.

Pure aspergillic acid melts at 93°; it is optically active ($[\alpha]_D = +14^\circ$). Analysis and molecular weight determination agree with the formula $C_{12}H_{20}O_2N_2$. It possesses a hydroxyl group, which is responsible for its acidic character (pK 5.5); the other oxygen atom could not be derivatized. Its ultraviolet absorption spectrum shows a characteristic maximum at 325 m μ . It can be distilled with steam or *in vacuo* without loss of biological activity, and it is remarkably stable under extreme conditions of acidity and alkalinity.

The high melting entity present in brown-sugar-containing media is a closely related substance (MP 149°, $[\alpha]_D = +42^\circ$) of the formula $C_{12}H_{20}O_2N_2$. Its biological activity is about $\frac{1}{10}$ of the activity of aspergillic acid of MP 93°.

The active substance elaborated by Glister's unclassified mold is unquestionably identical with aspergillic acid MP 93°, although the cultural characteristics of this mold are different from those of *Aspergillus flavus*.

SYNTHESIS OF PYRIDOXINE BY A "PYRIDOXINLESS" X-RAY MUTANT OF *NEUROSPORA SITOPHILA*. *J. L. Stokes, J. W. Foster and C. R. Woodward, Jr.*, Research

Laboratory, Merck & Co., Inc., Rahway, N. J.

A pyridoxine-requiring mutant of *Neurospora sitophila* grew normally in the absence of pyridoxine (vitamin B₆) if the culture medium was buffered with sodium acetate. On adjusting the medium to different pH levels, appreciable growth of the fungus occurred only at pH 5.8 or higher. At these pH values, it is also necessary to supply the mutant strain with ammonium compounds as nitrogen sources, other forms of nitrogen being unsuitable. Under these conditions, the ability to synthesize pyridoxine is restored. Other aspects of the pH-ammonium-nitrogen relationship to pyridoxine synthesis in the mutant and also some genetic implications are discussed.

SOME EVIDENCE ON THE ETIOLOGY OF CANCEROUS PROPERTIES AS EXEMPLIFIED IN PLANT CELLS. *Philip R. White and Armin C. Braun*, Department of Animal and Plant Pathology, The Rockefeller Institute for Medical Research, Princeton, New Jersey.

Tumors characterized by an unrestrained and discoordinate type of growth *in situ* and *in vitro*, by capacity to produce new tumors when transplanted into fresh hosts, and in at least one case by a markedly anaerobic type of respiration, yet free of any infectious agent either bacterial or virus in nature, have now been produced in three different and unrelated hosts: sunflower, tomato and periwinkle, by three different methods, involving inoculation with crown-gall bacteria and subsequent elimination of the infecting organisms. It has been possible to demonstrate the co-operation of some one or more growth-substances of the auxine type in the initiation of the cancerous change responsible for these tumors and to establish the fact that this cancerous change goes to

completion within a few hours and perhaps minutes after establishment of contact between the tumefacient agent and the host cell undergoing change. These observations greatly narrow the field in which we must look for the basis of this change.

AN ANALYSIS OF THE ANTAGONISTIC AND THE SYNERGISTIC ACTION OF ACETONE, ETHYL ALCOHOL, BUTYL ALCOHOL, CHLOROFORM, ETHER, AND URETHANE ON SULFANILAMIDE INHIBITIONS. *Frank H. Johnson, Henry B. Eyring, and Walter Kearns, Departments of Biology and Chemistry, Princeton University.*

According to the temperature, and to the concentrations employed, the sulfanilamide inhibition of luminescence may be greatly increased, greatly decreased or unaffected by the addition of urethane. The data indicate that these two inhibitors, which are known to act directly on the luminescent system, also enter into a loose, reversible combination with each other. The equilibrium constant of the sulfanilamide-urethane mutual adsorption or combination, which can be calculated from the data on luminescence, enables a fairly accurate, quantitative prediction of the effects of a wide range of concentrations of the two inhibitors mixed in various proportions. Furthermore, the theory predicts that, at suitable temperatures and concentrations, antagonism and synergism, respectively, may be expected in the inhibition of luminescence by sulfanilamide plus ether, alcohols, chloroform, acetone, and certain other substances. Data from experiments have amply verified these predictions. Although luminescence lends itself to more direct and precise analysis than growth, experiments on the growth of several species of bacteria indicate that fundamentally the same effects are obtained. The phenomenon of loose combination of inhibitors with each other, as well as with a common protein, may also be significant in a variety of circumstances involving stimulation by low concentrations, drug antagonisms, drug fastness, *et cetera*.

THE ACTION OF AN ANTIBIOTIC SUBSTANCE (PENATIN) ON BACTERIOPHAGE. *Thomas*

F. Anderson, The Johnson Foundation for Medical Physics, University of Pennsylvania, Philadelphia, Pa.

In the presence of 0.1% glucose, 0.1 mg. of penatin per ml. reduces the concentration of plaque-forming particles of anti-coli phage to $\frac{1}{1000}$ of its original value in 18 hours at room temperature while 0.001 mg. of penatin per ml. reduces the concentration to $\frac{1}{5}$ the original value in the same period. The penatin-inactivated phage particles retain their original morphology as seen in the electron microscope as well as their ability to adhere to susceptible bacteria. The penatin-inactivated virus suspension is also capable of inhibiting colony-formation of bacteria which are susceptible to lysis by the normal virus. This inhibitor of colony-formation is absorbed by the bacteria and the reaction may well be analogous to that observed recently by Luria and Delbrück in which phage suspensions partially inactivated by ultra-violet light were shown to have a similar effect. The significance of these findings in relation to the nature of viruses is discussed.

INCREASED INCIDENCE OF VIRUS INCLUSION BODIES IN HUMAN THROATS. *Jean Broadhurst, Estelle MacLean and Inez Taylor, Teachers College, Columbia University, New York City.*

Examination of another series of throat specimens for the inclusion bodies reported earlier by our laboratory was suggested by the prevalence of respiratory affections in our area this winter.

Throat smears of 224 unselected students in attendance in college and nursing classes between January 17 and March 15, 1943, were positive for these inclusion bodies in 35 to 87 per cent of the four groups examined or in 65 per cent of the whole number. This is a marked increase over the 8 per cent reported for 250-odd students in 1936. The positive 1943 specimens showed also a greater number of affected cells—18 per cent of the specimens being heavily affected in contrast to less than 1 per cent in 1936.

Incidentally, the Bond-Mann stain used for identifying the Negri bodies of the rabies virus gives the same differential (red) stain for these throat inclusion bodies; and in the

larger bodies, especially in specimens from long-period carriers, the stippled or composite character of these bodies is clearly observable.

PREPAREDNESS FOR DEFENSE AGAINST INFLUENZA. *Ward J. MacNeal and Ernestine R. Parker*, Department of Bacteriology, New York Post-Graduate Medical School and Hospital (Columbia University).

Influenza appears to be caused by a readily transmissible virus which develops in the superficial respiratory mucous membranes, predisposing them to penetrating bacterial invasion. Fatigue, intoxication, inhalation of dust and exposure to inclement weather increase susceptibility. Crowded quarters encourage mass infection sufficient to overcome natural resistance. In ferrets and mice, air-borne transmission is related to quantity of virus.

Brief immunity follows recovery in ferrets and the mucous membrane again becomes susceptible while the blood still contains neutralizing antibodies. Vaccination promises only relative protection. Where facilities for air-conditioning are available the virus may be destroyed by ultraviolet radiation or by minute quantities of glycols.

We have found that the virus, dried in mucin on a glazed surface, may remain potent for 45 days and, dried on the soap-free skin of the hand, for at least forty minutes. It is quickly inactivated by soap and by lysol. Commonly used mild antiseptics, as for example liquor antisepticus, inactivate the influenza virus in saliva in 30 seconds. Hence the use of these agents

in toilet of the hands, face, mouth, nose and throat is recommended.

TEST OF ANTI-DYSENTERY AGENTS IN EMBRYONATED EGGS. *Ward J. MacNeal, Anne Blevins and Marcello Pacis*, Department of Bacteriology, New York Post-Graduate Medical School and Hospital (Columbia University).

Embryonated eggs inoculated with decimal dilutions of dysentery cultures were treated by the simultaneous or sometimes subsequent injection of therapeutic agents, including bacteriophage, sulamyd, sulfathiazole, sulfaguanidine and control broth. The eggs were candled daily and the dead ones examined and cultured promptly. The chicks which hatched were sacrificed and cultured. Many hundreds of eggs have been used. Only a general preliminary statement can now be given.

In the earlier experiments with Sonne strains the total survivals four days after inoculation were as follows: bacteriophage 60 per cent, sulamyd 55 per cent, sulfathiazole 49 per cent, sulfaguanidine 38 per cent and control broth 19 per cent. Survivals to maturity within the shell twelve days after inoculation were in percentages, respectively, as follows: 48, 46, 44, 19, 10. Live chicks hatched were, respectively, 28, 24, 35 (sulfathiazole), 11 and 2 per cent.

As a rule cultures failed to recover bacteria but did recover phage from the phage-treated specimens and cultures of those treated with the sulfonamides frequently recovered the bacteria even when the chicks actually hatched. Further studies with other dysentery cultures have given somewhat similar results.

PERSISTENCE OF THE VIRUS OF ST. LOUIS ENCEPHALITIS IN THE CENTRAL NERVOUS SYSTEM OF MICE FOR OVER FIVE MONTHS

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Many demonstrations of persistence of viruses in the tissues of various animal hosts have been recorded (Olitsky and Long, 1929). As a rule, the presence of these infectious agents has been a sequel to clinically manifest disease in the host concerned. The prolonged incubation periods which may follow the peripheral injection of rabies (street) virus or the intracerebral inoculation of the virus of Borna disease are special instances of an apparently different nature. Evidence offered herewith seems to show that under certain conditions the virus of St. Louis encephalitis may invade the central nervous system (CNS) of susceptible mice and remain dormant there for considerable periods of time.

Relatively few instances of recovery of neurotropic viruses from the CNS after extended periods subsequent to an initial infection have come to the present writer's attention. Da Fano and Perdrau (1927), and Perdrau (1938) claimed to have detected herpes virus in the brains of rabbits up to several months after experimentally induced infections. The specific nature of the recovered agent was not demonstrated, however, and the evidence that herpes virus had actually been retrieved rested upon the clinical syndrome and incubation period in passage animals. Olitsky, Rhoads and Long (1929), using the technic of cataphoresis to concentrate virus from a filtrate of spinal cord, succeeded in demonstrating poliomyelitis virus in a monkey 26 days after intracerebral inoculation, at a time when the animal had almost completely recovered from the resulting paralysis. Webster and Clow (1936a), working with St. Louis encephalitis virus in mice, state that from a single animal of a resistant strain they were able to recover virus 4 weeks after it had been dropped into the nares. Theiler (1937) has been able to show that the virus of an enzootic encephalomyelitis of mice is present in the brain and spinal cord over a year after recovery from the clinical disease.

It has been possible to demonstrate that mice, passively protected against the virus of St. Louis encephalitis, may continue to harbor the virus for several months after it has been dropped into the nares. Mice passively immune to various viruses may be obtained by breeding them of mothers that have been actively immunized. The antibody transferred from mother to young persists for several weeks after birth. In the course of experiments designed to test the protection thus afforded the progeny of immune mothers against St. Louis encephalitis virus instilled intranasally when the mice were 2 weeks of age (Slavin, Hale, and Berry, to be published), a certain proportion of the animals survived a lethal dose of virus. On the other hand, control animals died regularly within

8 days after receiving a similar inoculum. It was noted that a few of the passively-protected survivors developed encephalomyelitis after prolonged periods of incubation—from 14 to 33 days—at a time when antibody acquired from the mother was in all probability rapidly diminishing. This observation made it seem reasonable to inquire whether, of the survivors who remained well, a few might continue to harbor the virus.

To test the point, 28 mice were available. All had been born of mothers hyperimmunized to St. Louis encephalitis virus (Freeman strain), and had received intranasally 100 minimal cerebral lethal doses of the same strain when they were 2 weeks of age. Frequent inspection had disclosed no clinical evidence of disease of the nervous system. At irregular intervals 2 of the mice were killed by exsanguination from the heart under ether anesthesia. The brains and spinal cords of the pair were triturated as a pool, and the supernatant fluid obtained by low-speed centrifugation was used to inject from 6 to 15 stock mice intracerebrally. Serial passage was not done. Of the 14 pairs passed in this fashion, the CNS of 3 pairs proved to be infective, producing the typical syndrome of encephalomyelitis. Ten-per cent suspensions of the brains of the infected passage animals were used for intracerebral inoculation of both stock mice and mice that had, as a result of immunization, previously displayed a solid cerebral immunity to the virus of St. Louis encephalitis. Without exception, second passages resulted in the death of stock mice from encephalomyelitis within 4 or 5 days, the time usually required for a 10-per cent suspension of mouse brain antigen of the Freeman strain to kill the mice after inoculation by the intracerebral route. Immune mice, on the other hand, remained well indefinitely. This fact serves to establish the identity of the virus. The 3 successful demonstrations of the presence of St. Louis encephalitis virus in the CNS were accomplished 71 days, 106 days, and 162 days after intranasal inoculation. In the 11 failures, the lapse of time between intranasal inoculation and passage varied from 55 to 162 days. Of the animals in which the CNS was shown to contain virus, the spleens and nasal mucous membranes of 2 pairs were also tested for virus activity, but with only negative results. The lungs, spleens, and nasal mucous membranes of 2 pairs whose central nervous systems proved non-infective were also passed by intracerebral injection into susceptible mice, again with negative results.

All the mice used in the present experiments were from an inbred stock of the Swiss strain among which spontaneous encephalitis has been singularly absent. Blind passages of the CNS of stock mice have been carried out on 2 occasions, on each of which passage was performed at weekly intervals through 6 brain-to-brain transmissions. No infectious agent was obtained from the brains and spinal cords of stock mice.

DISCUSSION

The apparent latency of infection of the CNS of mice of a susceptible strain by the virus of St. Louis encephalitis finds an analogy in some experiences of Bedson (1929) with psittacosis virus. From the spleens of mice inoculated with apparently neutralized mixtures of virus and antiserum, or from the spleens of those

inoculated with a small dose of active virus after previous immunization with formalinized virus, Bedson was able to obtain the infectious agent after a lapse of several months, although the infected animals remained well throughout that period. In his experiments, as in that herein reported, it appears that virus initially restrained by the presence of immune bodies ultimately achieves a nice balance with inhibiting factors of the host, is able to survive, and in all probability to multiply to some extent.

The carriage of St. Louis encephalitis virus by arthropod vectors may be important in the transmission of the disease to man. Recently, Hammon (1943) has shown *Culex tarsalis* to be a naturally infected host of the virus, and has demonstrated that this, as well as other species of mosquitoes, are capable of transmitting the St. Louis infection in the laboratory. It is suggested by Hammon that, since *Culex tarsalis* winters as an adult, it may serve to carry the virus over from one season to another. Although this author also found that the serums of a variety of vertebrates present in an endemic focus of the disease contain neutralizing antibodies against the St. Louis virus, the agent has not thus far been recovered from any other naturally infected vertebrate host than man.

The demonstration herein recorded of the persistence of St. Louis encephalitis virus in the CNS of a mammalian host suggests the possibility that vertebrate reservoirs may exist. Continued harborage of the virus in tissues other than the CNS has not been demonstrated, but Webster and Clow (1936b) have shown that the virus is capable of multiplication in the spleen of the mouse, and Lennette and Smith (1940) have propagated it in mouse testicle. Whether or not the virus harbored for a long period of time within or outside the CNS is capable eventually of reaching the circulation where it would be available to an arthropod seeking a blood meal, and whether in doing so it may exist in a state in which it is able to endure within an invertebrate vector are not known.

SUMMARY

Under certain conditions, the virus of St. Louis encephalitis occasionally produces subclinical infection of Swiss mice. The virus has been recovered from the central nervous system of such mice as late as 162 days after instillation into the nares.

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A COMPARISON OF THE VALUE OF THE AGGLUTINATION AND PRECIPITIN REACTIONS IN THE SEROLOGICAL TYPING OF GROUP A STREPTOCOCCI¹

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Two methods, slide agglutination (Griffith) and type-specific precipitin tests (Lancefield), are now being used for routine typing of Group A streptococci. Agglutination as a means of type differentiation was first established on a firm basis by Dochez, *et al.* (1919). It was not, however, until Griffith (1926, 1935) published his work on slide agglutination that interest in typing hemolytic streptococci became widespread. Previously, macroscopic agglutination tests had been employed in studying streptococci; but the difficulties encountered in obtaining stable, diffuse suspensions had prevented their general use. Griffith's method not only reduced these difficulties but also the time and material required.

While Griffith merely attempted to determine type-specificity by revealing the dominant antigen concerned in the agglutination reaction, Lancefield (1940-41) has made more detailed studies of the antigenic structure of hemolytic streptococci. This investigator has shown that at least two antigenic factors, designated M and T, are concerned in the type-specific reactions of Group A streptococci. The M factor, a type-specific protein substance, is present in matt and mucoid variants. Immunization of rabbits with these variants gives rise to anti-M precipitins and type-specific protective antibodies. While the T factor is apparently the important reagent concerned in the agglutination reaction, many types are agglutinated by the M antibody. Although the presence of two different type-specific factors in a single microorganism suggests the possibility of various antigenic combinations, in most instances M and T correspond as to type.

For the past six years the relationship of Group A hemolytic streptococci to rheumatic fever has been studied at Irvington House, a sanatorium for rheumatic children. During this period an attempt was made to type all Group A streptococci isolated from carriers as well as those associated with upper respiratory infections. Since the majority of observers have relied upon slide agglutination, this method alone was employed at the beginning of this study. Although the sera used for slide agglutination were absorbed to remove all non-specific agglutinins for stock strains of all types, cross reactions were often encountered in testing recently isolated cultures. The precipitin reaction was therefore employed to check the slide agglutination findings. The precipitin reaction has been of value not only in determining the type of many strains which showed negative or equivocal results by slide agglutination reaction, but also in establishing the existence of several previously unidentified types.

¹ This work was aided by a grant from the Commonwealth Fund.

EXPERIMENTAL METHODS

Preparation of agglutinating and precipitating sera

Strains of known types for immunizing rabbits were either obtained from Dr. Griffith or had been checked by him.

Preparation of vaccines. 100 ml. of broth warmed to 37°C. was inoculated with 30 ml. of rapidly-growing young culture. After 3 hours incubation the culture was killed by adding sufficient boiling physiological saline to bring the temperature to 56°C. and was then maintained at this temperature in a water bath for 15 minutes. The vaccine was centrifuged and resuspended in 125 ml. of physiological saline.

Immunization of rabbits. Rabbits were immunized over a period of from 8 to 10 weeks with vaccines prepared from young heat-killed cultures. Matt or mucoid variants were used in preparing all vaccines since the primary objective of the immunization of rabbits was the preparation of anti-M precipitating sera. Agglutinating sera were often obtained, not only from rabbits who showed an anti-M precipitin response but also from those in whose sera no type-specific precipitins were demonstrable. Following an initial injection of 1 ml. of vaccine the dose was gradually increased until the rabbits received as much as 8 to 16 ml. in a single injection. The rabbits were injected on three successive days each week for three weeks and then bled four days following the last injection. Thereafter they received injections five days a week on alternate weeks; and test bleedings were taken four days following the last injection of each weekly series. The serum was tested for agglutinins and for anti-M precipitins. When the test bleeding showed a 2 to 3 plus reaction with the homologous M extract, or a slide agglutination titer of 1:80 or higher, large bleedings were taken the following day. All sera were absorbed before use.

Absorption of sera

Sera for slide agglutination. It was usually found that agglutinating sera warranted absorption only if the titer for the homologous type was higher than that for any heterologous type. A strain of type 24 (Griffith, Sylvia Turton) which had lost its type-specificity was used for absorption. Three to four parts of undiluted serum were absorbed with one part of heat-killed organisms for 2 hours at 37°C. In most instances, absorptions were repeated until the sera no longer agglutinated suspensions of type 24. They were then tested against several representative strains of all known types and absorbed with those heterologous strains which were agglutinated. From one to nine absorptions were necessary to remove all cross reactions for stock cultures. In many instances, multiple absorptions with a single type were required. A number of the recently prepared sera were found to be satisfactory after a single absorption with living cultures. Therefore, a few of these sera were absorbed with both living and heat-killed microorganisms. The results suggested that a single absorption with living cultures was as effective as multiple absorptions with heat-killed microorganisms of the same type. Sera for slide agglutination were

diluted 1:5 or 1:10, depending upon the titer of the sera after absorption. Those sera which were absorbed with living cultures were filtered through a Seitz filter.

Sera for precipitin tests. Four parts of serum were mixed with one part of packed heat-killed Group A streptococci of a heterologous type, incubated for 2 hours at 37°C., stored in the ice box overnight, and centrifuged the following day. To determine their specificity, the absorbed sera were tested with homologous M extracts from which the Group A carbohydrate had been removed by alcohol precipitation, with heterologous M extracts, and with Group A carbohydrate. When non-specific precipitins were found following a single absorption, the serum was reabsorbed. Occasionally a serum, which showed strong reactions with a number of heterologous M extracts, failed to react with the homologous M extract after absorption, hence was discarded. Serum from which all non-specific precipitins had been removed were filtered through a Seitz filter; then merthiolate was added in a final concentration of 1:10,000.

It was suggested by Eisman (1940) that a single lot of microorganisms could be employed for several absorptions. After being used, the bacterial sediment was washed twice, resuspended in physiological saline, and heated in a water bath at 100°C. for 1 hour in order to destroy any antibody adhering to the bacterial cell. Although bacteria heated in this manner did not prove wholly satisfactory in absorbing sera for the slide agglutination reaction, it was found that they could be used as often as five times in absorbing sera for the precipitin reaction.

Slide agglutination

Suspensions for slide agglutinations were prepared from cultures grown 18 hours either in meat-infusion neopeptone broth with 0.1 per cent NaHPO_4 or in Griffith's trypsin broth (Pauli and Coburn, 1937). The organisms were packed by centrifugation, the supernatant was poured off, and the bacterial sediment was broken up with a capillary pipette. Drops of the suspension were placed on a slide divided into approximately quarter inch squares with a china marking pencil. The sera² were added and mixed with a 1 mm. 28 gauge platinum loop. The slide was rotated, examined with a hand lens, and the agglutination was recorded. When no agglutination occurred, the suspension was stored at room temperature and retested the following day. In many cases, agglutination of a culture appeared to be inhibited by a capsular substance which could be removed by aging at room temperature (Keogh and Simmons, 1940). When a suspension was granular or tended to agglutinate spontaneously, a number of methods were employed in an attempt to obtain workable suspensions. In some instances, satisfactory results were obtained by short periods of incubation or rapid transplants of the cultures; in others, by growing the cultures in broth containing 10 per cent horse serum or 10 per cent active trypsin (Coburn and O'Connell, 1939).

² I am indebted to Drs. Lancefield and Griffith, and to the Lederle Laboratories for many of these sera.

Precipitin tests

M extracts were prepared from the sediment of cultures grown 18 hours in 500 ml. of broth* (Lancefield, 1928). It was found essential to use a highly nutrient well-buffered medium in preparing M extracts for precipitin tests. Since non-type-specific precipitins were removed from the sera, removal of the group-specific polysaccharide from crude extracts by alcohol precipitation was omitted. 0.05 ml. of extract was pipetted into small tubes (5 by 50 mm.) and 0.05 ml. of absorbed type-specific precipitating serum⁴ was added. Controls of each serum were made with type-specific extract. The tests were read within 15 minutes and after 2 hours at room temperature, and were considered definitely positive only when the reactions with the extracts being tested were as strong as those with the controls. Confirmatory tests with 0.4 ml. of extract and 0.2 ml. of serum were done when the reactions with the extracts being tested were weaker than those with the controls. Reactions with a new lot of serum were considered unreliable until the sera had been used in testing a large number of recently isolated strains of heterologous types. Sera for types 9, 11, 13, and 27 showed marked cross reactions with heterologous extracts even after absorption and were considered unreliable. Since no strains of type 8 were encountered, the value of type 8 serum was unknown. No satisfactory anti-M serum has been prepared for type 24.

Grouping

Hemolytic streptococci were established as members of Group A (Lancefield, 1933) before attempts were made to type them. Polysaccharide extracts were prepared by extraction with either hydrochloric acid (Lancefield) or with formamide (Fuller, 1938).

TYPE DETERMINATIONS

Attempts were first made to type all Group A strains by the slide agglutination method. M extracts were prepared from strains which showed positive agglutination reactions; and precipitin tests were set up with anti-sera for the corresponding types. Since 1939, whenever a strain could not be typed by this procedure, precipitin tests were done with all types for which sera were available. While several factors may be responsible for the agglutination reaction a single antigen, the M substance, is responsible for the type-specific precipitin reaction which parallels the protective antibody response. Therefore, whenever the results obtained by slide agglutination and precipitin tests differed, type determinations were based upon the precipitin reaction. To date, only one strain of Group A hemolytic streptococcus has been reported in which type determination based upon the agglutination reaction differs from that based upon the

* The broth used in these experiments was a modification of that described by Todd and Hewitt (1932). Neopeptone was used and lean beef was substituted for horse meat. The medium was rendered sterile by filtration through a Berkefeld filter.

⁴ I am indebted to Dr. Lancefield for many of these sera.

precipitin reaction (Lancefield, 1940). In the course of this study two groups of strains which appeared to contain the M substance characteristic of one type and the T substance characteristic of another were encountered. These strains have been designated type 10-12 and type 27 (M485).

Type 10-12

Six strains were isolated which appeared to be type 12 by agglutination and type 10 by precipitin test. At the time of their isolation, no type 12 precipitating serum was available. Subsequently, however, a type 12 precipitating serum was obtained from Lederle Laboratories. This serum was indistinguishable from type 10 by precipitin test but was specific for type 12 by agglutination. This group of strains is being studied by Watson and Lancefield (1940).

Type 27 (M485)

Hemolytic streptococci which were agglutinated by type 27 serum were isolated from 72 children. Sixty-two strains were isolated in the winter of 1939-40 (Kuttner and Krumwiede, 1941) and 10 in the summer and fall of 1940. Slide agglutination reactions with many of these strains were weak or showed cross reactions with types 11 and 28. Since no reliable type 27 precipitating serum was available, rabbits were immunized with a strain M485 isolated from a child with pharyngitis, and with type 27 (Griffith's 780 Tate). Precipitating and agglutinating serum was obtained from the rabbits immunized with strain M485 while agglutinating serum only was obtained by immunization with strain 780 Tate. Reciprocal absorption of the agglutinating sera established strain M485 as type 27 (agg.).⁵ Sera against strain M485 gave positive precipitin reactions with extracts of all 72 strains isolated but failed to react with extracts of strain 780 Tate. The failure of extracts of this strain of type 27 (agg.) to react with anti-M serum prepared from a heterologous strain of type 27 may be explained in one of two ways. Since strain 780 Tate did not elicit an anti-M response in rabbits, it may have contained little or no M substance. On the other hand, all strains of streptococci belonging to type 27 (agg.) may not have a common M substance. In view of this possibility, these 72 strains have been classified as Type 27 (M485).

Provisional new types

Many strains were encountered which could not be typed by precipitin tests. Slide agglutination reactions with many of these were negative or equivocal, with the sera available. Since positive precipitin tests were considered the criteria for type determination in this study, these strains were not immediately classified. Such unclassified strains which appeared to be of special interest were used to immunize rabbits. The sera were tested for homologous agglutinins and precipitins both before and after absorption; and those strains against which specific anti-M precipitating sera were obtained were considered provisional

⁵ The qualifying term (agg.) indicates that type determination in this instance was based upon the agglutination reaction only.

new types. To date six provisional types have been identified and designated tentatively as provisional⁶ types 32 (formerly C51), 33 (formerly R31), 34, 35, 36 (formerly B35) and 37 (Kuttner and Krumwiede, 1941; Kuttner and Reyersbach, 1943). Specific agglutinating sera have been obtained for only two of these: viz. 32 and 36. The tests to determine the specificity of one of these types are presented in table 1. Dr. A. T. Wilson of The Hospital of the Rocke-

TABLE 1
Tests for specificity of provisional type 32

TYPE	PRECIPITIN REACTION		AGGLUTINATION REACTION	
	Unabsorbed serum	Serum after absorption with type 11	Unabsorbed serum diluted 1:10	Serum after absorption with type 24 diluted 1:5
1	±	—	+	—
2	±	—	—	—
3	—	—	—	—
4	—	—	—	—
5	—	—	—	—
6	—	—	—	—
8	—	—	—	—
9	—	—	+	—
10	±	—	—	—
11	—	—	—	—
12	—	—	+	—
13	—	—	—	—
14	±	—	—	—
15	—	—	±	—
17	+	—	—	—
18	+	—	—	—
19	±	—	—	—
22	±	—	—	—
23	—	—	++	—
24	—	—	+++	—
25	—	—	+++	—
26	±	—	++	—
27	—	—	—	—
28	—	—	—	—
29	—	—	—	—
30	±	—	—	—
32*	+++	+++	+++	+++

* Provisional new type.

feller Institute has kindly supplied us with sera for 4 additional provisional types designated as provisional types 38, 39, 44, and 45. After absorption the sera for prov.⁷ types 38 and 39 contained type-specific agglutinins. The sources of 102 strains belonging to these provisional types are presented in table 2.

⁶ The provisional numbers designating these newly recognized types have been assigned as a matter of expediency after conference by Dr. Ann G. Kuttner, Director of Irvington House, with Drs. H. F. Swift and R. C. Lancefield of The Hospital of the Rockefeller Institute.

⁷ Prov. is the abbreviation used for provisional in the remainder of paper.

Results of typing

Of 382 strains tested, 333 (87 per cent) were typed by anti-M precipitin tests. A summary of the slide agglutination reactions of these strains is shown in table 3.

TABLE 2
Sources of provisional new types

PROVI- SIONAL TYPE*	SOURCE OF STRAINS USED FOR IMMUNI- ZATION	ISOLATED AT IRVINGTON HOUSE			TOTAL	NO. OF STRAINS EN- COUNTERED ELSEWHERE
		No.	Source	Date		
32	U.R.I.	{ 1 14 12	Adm. carrier Carriers U.R.I.	May '37 June '37-Mar. '38 Jan. '38-May '38	29	2
		1 1	Adm. carrier Carrier	June '38 July '39		
36	U.R.I.	{ 1 18 6	Adm. U.R.I. U.R.I. Carriers	Oct. '41 Oct. '41-Jan. '42 Dec. '41-Mar. '42	26	0
		1	Adm. carrier	June '39		
38	U.R.I.	1	Adm. carrier	July '39	3	†
		{ 1 1	Adm. carrier Carrier	Sept. '40 Oct. '40		
39	U.R.I.	1	Adm. carrier	Sept. '39	1	†
46	U.R.I.	{ 1 1	Adm. carrier Carrier	Oct. '41 Oct. '41	2	†
33	Carrier	{ 1 22 1	Adm. carrier Carriers U.R.I.	June '39 Sept. '39-Jan. '40 Oct. '39	27	5
		1	Carrier	Aug. '40		
		1	Adm. carrier	Sept. '40		
		1	U.R.I.	Sept. '40		
34	Carrier	1 1	Adm. carrier Adm. carrier	May '40 Oct. '40	2	0
35	U.R.I.	2	U.R.I.	Feb. '37	8	1
		{ 3 3	Carriers U.R.I.	July '41-Aug. '41 Aug. '41-Sept. '41		
45	U.R.I.	1	Carrier	Nov. '41	1	†
37	Carrier	3	Carriers	Jan. '42-Apr. '42	3	0

U.R.I. = Upper respiratory infection. Adm. = On admission. Bracket indicates probable spread.

* See footnote page 122.

† These types were established at The Hospital of the Rockefeller Institute.

Two hundred and three strains (61 per cent of the typed strains) were correctly typed on the basis of the agglutination reaction; while 20, which reacted with a single agglutinating serum, and 14 of the 74, which reacted with two or more agglutinating sera, would have been incorrectly typed had no precipitin tests

been done. Thirty-six strains failed to react with any agglutinating sera. Although 297 strains (78 per cent of those typed by the precipitin reaction) could

TABLE 3
Comparison of slide agglutination and anti-M precipitin reactions

(1)	(2)	(3)	(4)	(5)	(6)	(7)
ANTI-M PRECIPITIN REACTION	SLIDE AGGLUTINATION REACTIONS					TOTAL STRAINS
	Type specific	Non-type specific	Multiple agglutinations		Nega- tive	
			Type specific strongest	Non-type specific equal to or stronger than type specific		
			strains	strains		
Types designated by Griffith						
Type 1.....			3 (spon. agg.)			3
Type 2.....	6					6
Type 3.....					2	2
Type 4.....	38		9 (types 3, 6, 8)†	2 (types 24, 26)†		49
Type 5.....	4					4
Type 6.....	5				1‡	6
Type 10-12 ..	3*		3 (type 11)†*			6
Type 14.....	2	1 (type 28)†			1	4
Type 15.....	43					43
Type 17.....	1		1 (types 15, 19)	4 (types 15, 19, 23)		6
Type 18.....	4	1 (type 22)‡			5	10
Type 19.....		1 (type 23)		2 (types 15, 17, 23)		3
Type 22.....			1 (type 6)		1	2
Type 23.....				1 (types 15, 17, 19)	1	2
Type 25.....	1					1
Type 26.....	3		4 (type 4)			7
Type 27 (M485)	49		22 (types 11, 28)	1 (types 11, 28)		72
Type 28.....	1					1
Type 29.....	1			1 (type 4)		2
Type 30.....				2 (types 5, 15, 17)		2
Provisional new types						
Agglutinating sera available						
Type 32.....	14		14 (types 5, 15)	1 (types 5, 15)		29
Type 36.....	26					26
Type 38.....	1	1 (types 23)	1 (types 5, 27)			3
Type 39.....	1					1
Type 46.....			2 (types 4, 24, 26)			2
No agglutinating sera available						
Type 33.....		15 (type 8)‡			12	27
Type 34.....		1 (type 28)‡			1	2
Type 35.....					8	8
Type 37.....					3	3
Type 45.....					1	1
Total	203	20	60	14	36	333

Spon. agg. = spontaneous agglutination.

* Agglutinated by Type 12 serum.

† Types of sera causing the commonest cross reactions are shown in brackets.

‡ Grown with trypan.

apparently be typed by slide agglutination, an error of at least 12 per cent would have occurred if no precipitin tests had been done.

Strains agglutinated by serum of one type only

Although extracts of 203 of the strains which were agglutinated by serum of one type only reacted with anti-M serum for the same type, extracts of 20 others reacted with anti-M serum of other types (table 3, Column 3). These results suggested that these 20 strains might contain the M substance characteristic of one type and the T substance characteristic of another; further evidence, however, indicated that the agglutination reactions of these strains were non-type-specific. For instance, many strains of prov. type 33 tended to agglutinate with type 8 serum after growth in the presence of active trypsin but failed to show a positive precipitin reaction with type 8 serum. Since the value of the type 8 serum for the precipitin reaction was not known, a type 8 agglutinating serum with a higher titer (positive in a dilution of 1:320) was prepared. This serum failed to agglutinate any of the prov. type 33 strains even in low dilutions. The agglutination reactions with prov. type 33 cultures previously obtained with the stock type 8 agglutinating serum were therefore considered non-specific.

Repeated absorption had weakened the sera for types 14, 18, and 19. Furthermore, it had been impossible to remove all non-specific agglutinins from many of the sera. Agglutination reactions were therefore considered non-specific when a strain, belonging to a type for which the agglutinating serum was weak, was agglutinated only by a serum known to contain non-specific antibodies.

Strains agglutinated by serum of two or more types

Many investigators have noted the tendency of some types to show cross reactions when tested by slide agglutination. Plummer (1941) and Rudd, *et al.* (1939) had difficulty in distinguishing between types 17 and 23, Neisser (1939) and Coburn and O'Connell (1939) between types 15 and 17, and Keogh and Simmons (1940) between types 15, 17, and 23. During the course of this study, although strains of type 15, isolated during an outbreak of pharyngitis, were agglutinated by type 15 serum only, the majority of strains of types 17 (ppt.),^{*} 19 (ppt.), and 23 (ppt.) showed a tendency to agglutinate with sera for types 15, 17, 19, and 23. Since some investigators have been able to differentiate these four types, it seems possible that they contain type-specific agglutinogens as well as agglutinogens common to two or more of these types.

Type 4 (ppt.), 26 (ppt.) and prov. type 46 (ppt.) form a second group of organisms which appear to contain a common agglutinin (Plummer, 1941; Rantz, 1942). Many strains belonging to these types tended to show cross agglutination reactions with sera for types 4, 24, 26 and the prov. type 46 established by Wilson. Attempts to eliminate these cross reactions from the serum for prov. type 46 resulted in the loss of all agglutinins.

The results of slide agglutination tests with strains belonging to these two categories (types 15, 17, 19 and 23; and types 4, 24 and 26) were difficult to interpret. The agglutination reactions with sera for two or more types were often equally rapid and marked. Repeated slide agglutination tests with different subcultures from a single strain often gave variable results.

* The qualification "ppt." indicates type determinations based upon the precipitin reaction.

Many strains belonging to prov. type 32 showed cross agglutination reactions with serum for types 15 and a few with serum for type 5. As may be seen in table 2, prov. type 32 serum failed to agglutinate type 5 strains and agglutinated type 15 strains only feebly before absorption. Since no prov. type 32 cultures were available at the time the types 5 and 15 sera were absorbed, these sera could not be tested or absorbed with prov. type 32. Subsequently a serum for type 15 has been absorbed with prov. type 32 without reduction of the titer for type 15.

Negative agglutination

Of the 36 strains (table 3, column 6) which could be identified by the precipitin reaction but failed to agglutinate with any sera, 25 belong to types for which no agglutinating serum was available and 9 to types for which the agglutinating serum was known to be weak. No attempt has been made to determine whether

TABLE 4
Agglutination reaction of strains which could not be typed by the precipitin test

AGGLUTINATION REACTION (SERA)	NUMBER OF STRAINS
Type 1.....	1
Types 4-24-26.....	5
Types 11-27-28.....	6
Type 12.....	2
Type 13.....	5
Types 15-17-19-23.....	2
Type 25.....	4
Prov. type 32.....	4
Irregular results.....	5
	15
Total	49

these 9 cultures represent strains containing the M substance of a known type in conjunction with T substance of an unknown type. The failure of a strain of type 23 (ppt.) to agglutinate may be due to lack of specific agglutinins for type 23 as opposed to agglutinins common to types 15, 17, 19, and 23 in the sera used. A granular culture of type 6 failed to agglutinate after growth in the presence of active trypsin.

Unclassified strains

Forty-nine strains could not be identified by the precipitin reaction with any of the anti-M sera available (table 4). Twenty-nine of these strains were agglutinated consistently by sera for one or more types. It is not known whether these strains contain little or no M substance or represent organisms containing an unidentified M substance in combination with a known T substance.

One strain was agglutinated strongly by type 1 serum. The organism formed glossy colonies and probably contained little M substance.

Five strains were agglutinated by sera for types 4, 24, and 26. The strength of the reactions with the different sera varied in different tests. The type 26 reaction was encountered least often and was weakest. One of these strains was isolated from a carrier who subsequently developed pharyngitis due to type 4 (ppt.).

Six strains were agglutinated by types 11, 27, and 28. Since these strains failed to react with type 27 (M485) or type 28 anti-M sera, and because reliable type 11 anti-M serum was not available, they could not be identified satisfactorily. One of these strains was isolated from a carrier who subsequently developed pharyngitis due to type 27 (M485). Since both strains were agglutinated by sera for types 11 and 27, they were difficult to differentiate on the basis of the slide agglutination reaction. A type 27 serum, however, prepared with a strain of type 27 (M485) failed to agglutinate the carrier strain.

Two strains (155K and 426S) were agglutinated by type 12 serum. Extracts of these cultures failed to react with antisera for the M substance common to types 10 and 12. Sera from a rabbit immunized with one of these strains showed specific agglutinins but no anti-M precipitins. Slide agglutination tests with dilutions of this serum and two other type 12 sera were done using suspensions of strains 155K, 426S, type 12 (SF42) and heterologous types. Strains 155K and 426S were indistinguishable from type 12. Although no reciprocal absorptions have been done, these strains have been classed as type 12 (agg.). They probably contain the T substance characteristic of type 12, and may either have an M substance distinct from that found in type 10-12 or be essentially glossy forms deficient in M substance.

Since no reliable type 13 precipitating serum for confirmatory tests was available, five strains which were agglutinated by type 13 serum have been tentatively designated type 13 (agg.).

Cross reactions among types 15, 17, 19, and 23 have made these four types difficult to differentiate by the slide agglutination technique. Although 9 strains showing cross agglutination reactions with these sera were identified by precipitin tests, two strains recorded in Table IV could not be specifically identified by either slide agglutination or precipitin tests.

Type 25 serum agglutinated four strains but did not give positive precipitin reactions with them. The serum from rabbits immunized with one of these strains showed a high agglutination titer for the homologous organism and for type 25 but gave no precipitin reactions with extracts of either.

Four strains were agglutinated by prov. type 32 sera. The reactions, however, were so weak they were considered insignificant. The agglutination reactions of five other strains were too variable to identify the strain; finally, fifteen strains failed to agglutinate with any sera.

Carrier strains

The type of all hemolytic streptococci isolated from children, known to carry Group A strains, was checked once a month by the slide agglutination test. Precipitin tests were omitted unless the slide agglutination reaction or a change

in colony form suggested that a child harbored a different type. A number of children carried Group A streptococci upon admission and many became carriers during their stay in the institution. In accord with the findings of other investigators, cultures isolated from carriers, as compared to those isolated from patients with upper respiratory infections, often tended to agglutinate spontaneously or were inagglutinable. In many instances when such strains could not be typed by slide agglutination, satisfactory precipitin reactions were obtained.

DISCUSSION

An attempt was made to type 382 strains of Group A hemolytic streptococci both by the slide agglutination technique (Griffith) and by the anti-M precipitin reaction (Lancefield). Since several antigens may be involved in type-specific agglutination, whereas a single antigen, the M substance, is probably responsible for the precipitin reaction, all type determinations were based upon the precipitin test. Two hundred and thirty-one strains (60 per cent) were shown to be members of the types described by Griffith. Type classification of all unidentified strains which appeared to be of special interest was attempted by the immunization of rabbits with representative strains. The type specificity of six cultures was determined on the basis of the M precipitin reaction, and these strains were classified as provisional new types. Without the use of the precipitin reaction four of these six provisional new types could not have been established, because attempts to prepare satisfactory agglutinating sera proved unsuccessful. One hundred and two strains (27 per cent) were shown to belong to provisional new types established in this laboratory and in the laboratory of The Hospital of the Rockefeller Institute.

A comparison of the typing results obtained by the slide agglutination reaction and by the anti-M precipitin reactions shows that more definite conclusions could be drawn from the latter. Positive precipitin reactions were obtained with 333 strains (87 per cent), all of which could be readily typed on the basis of the anti-M reaction. On the other hand, while positive agglutinations were obtained with 331 of the 382 strains tested, many of the reactions were unreliable or equivocal.

Unreliable agglutination reactions were often due to sera which had been weakened by repeated absorption and to sera from which all non-type-specific agglutinins had not been removed. The later sera led to false results with three groups of strains: recently isolated strains which appeared to contain larger proportions of non-specific antigen than the stock strains, strains belonging to provisional new types, and strains which were tested repeatedly because they appeared to be inagglutinable. Cross reactions with strains belonging to provisional new types were due to the fact that these types had not been established at the time the sera were absorbed. Therefore, these cross reactions were not eliminated. It was found that unless the presence of capsules interfered with agglutination, non-specific reactions were as apt to be encountered as were specific reactions when repeated attempts were made to agglutinate apparently inagglutinable strains. Unreliable results were also encountered when cultures were

grown in broth containing active trypsin. While such growth was of help in obtaining agglutination with some strains, this procedure often induced non-specific reactions. Keogh and Simmons (1940) are also of the opinion that unreliable results may be obtained by this method.

It is generally recognized that certain types show a greater tendency to cross reactions than others, and that reciprocal absorption of sera for these types may remove type-specific as well as non-type-specific agglutinins. Plummer (1941) has suggested that some types are closely related in their agglutination reactions and may therefore be grouped together. Since the typing of Group A streptococci is of value not only in the study of streptococcal epidemics but also in the study of streptococcal immunity, it is advisable to differentiate all types carefully even though some may be closely related. In most instances this may be done on the basis of the M-precipitin reaction which closely parallels the protective antibody response. During the course of this study some children developed infections due to hemolytic streptococci showing agglutination patterns similar to those encountered with strains they had previously carried. In each instance, although the carrier strain could not be identified, precipitin tests showed that it did not contain the M substance present in the strain isolated during infection. Since anti-M precipitins and protective antibody response correlate, differentiating such strains on the basis of the anti-M precipitin reaction is important in studying streptococcal immunity. Moreover, while combining two or more types, such as types 4, 24, 26 and prov. type 46, may not necessarily detract from the value of individual epidemiological studies, it makes comparison of the results of different investigators unsatisfactory. For example, reports from various localities of a number of epidemics due to types 4-24-26 may lead to the assumption that a single type is causing a widespread epidemic, when, in reality, four types (4, 24, 26 and prov. type 46) may be responsible for scattered outbreaks.

Many of the unreliable and equivocal results encountered with slide agglutination reactions are eliminated by use of the type-specific precipitin test. This test not only removes the problems presented by granular and inagglutinable cultures but also makes it possible to differentiate those types which appear to contain closely related agglutinogens. Sera for use in the precipitin test require fewer absorptions and may be more accurately tested for non-type-specific reactions than those used for slide agglutination. Furthermore, correlation of anti-M precipitins and protective antibodies makes the precipitin test of greater value in studying streptococcal epidemiology. The simplicity of absorbing sera for the precipitin reaction, however, does not eliminate the need for testing each new lot of serum against extracts of all known types and of setting up adequate controls.

Although the precipitin test is of greater value than slide agglutination, the former cannot at present completely replace the latter. Glossy strains can seldom be typed by the precipitin reaction since they contain little or no M substance. Although glossy epidemic-inducing strains have not been reported, complete epidemiological studies require the typing of all carrier strains. Furthermore, large quantities of media and sera are required for routine typing by the precipitin reaction, and to date it has been found impossible to prepare reliable

precipitating sera for all types. Many laboratories will, therefore, find it necessary to continue using the slide agglutination reaction until a complete set of precipitating sera can be prepared, and until some method is devised whereby accurate precipitin tests can be done with small quantities of sera.⁹ In the meantime, slide agglutination results should be confirmed in some manner. Plummer (1935) suggests the use of macroscopic agglutinin absorption tests. Macroscopic agglutination, however, involves many of the same difficulties encountered in slide agglutination and measures the same antigens. Type-specific-precipitin tests for confirmation of slide agglutination reactions give results comparable to those obtained by use of the precipitin reactions alone and overcome the greatest objection to their use for routine typing, the quantities of sera required. When the precipitin test is used as a confirmatory test, the necessity for setting up each extract against sera of all types is eliminated. Furthermore, during streptococcal epidemics, only a limited number of strains, which appear to be the same type on the basis of the slide agglutination reaction, need be tested. For complete epidemiological studies of Group A streptococcal infections, therefore, the combined use of slide agglutination and anti-M precipitin typing appears at present to be advisable. By combining these two methods of typing, the greatest amount of reliable information can be obtained.

SUMMARY

1. The results obtained in testing 382 strains of Group A hemolytic streptococci by slide agglutination (Griffith) and type-specific M precipitin reactions (Lancefield) were compared. Although the antigen-antibody systems which govern the former are not necessarily identical with those responsible for the M precipitin reaction, there appears to be a high degree of correlation between the two systems.

2. 333 strains (87 per cent) were typed on the basis of the anti-M precipitin reaction.

3. The isolation of 6 provisional new types is reported; and the value of the precipitin reaction in establishing the specificity of these types is stressed.

4. The difficulties encountered in the slide agglutination reaction are discussed with the suggestion that some means of confirming slide agglutination results should be employed.

5. Precipitin reaction with type-specific M substance has been recommended as an accurate means of confirming type determinations based upon slide agglutination reactions.

6. The results of this comparative study indicate that if a simple and economical anti-M precipitin technique were available, the classification of most unknown strains would routinely be more accurately accomplished by the precipitin than by the slide agglutination method.

⁹ Dr. Homer F. Swift, of The Rockefeller Institute for Medical Research, reports (personal communication) that it is possible to prepare sufficient extract from 40 ml. of broth culture to carry out complete anti-M precipitin typing in capillary pipettes.

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THE PIGMENT PRODUCTION OF *ACTINOMYCES COELICOLOR* AND *A. VIOLACEUS-RUBER*¹

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INTRODUCTION

Some species of *Actinomyces* produce very striking pigments which have aroused the interest of many investigators. As a result a great deal of work has been done on the genus, but, unfortunately, failure to understand the nature of these pigments has led to considerable confusion on this subject. It has been long recognized that these pigments have indicator properties, and their similarity to litmus has been mentioned in the literature; they may therefore be one color on one medium and an entirely different color on a second medium. In spite of this fact, authors of species frequently fail to describe the medium on which they have grown their cultures; so that it becomes very difficult to tell whether varying descriptions apply to different organisms or all to the same one.

Some students of the genus even take the point of view that all cultures producing a litmus-like (*i.e.*, blue-red) pigment form a single species. The present series of experiments were planned to investigate this point, the study beginning with two pigment-producing strains, isolated from soil, which superficial examination indicated to be quite different, although both produced pigments that were blue under alkaline conditions and red when acid. A comparison of these with other cultures and with descriptions in the literature yielded some interesting results.

REVIEW OF LITERATURE

Some of the most detailed investigations on this subject were made by Müller (1908), one of the earliest workers in this field. He described in detail an organism producing a blue pigment on potato which he called *Streptothrix coelicolor*. This organism is now described in Bergey's Manual as *Actinomyces coelicolor*. Müller, however, did not stop with a description of the organism. He also extracted the pigment by washing it out of potato with water and studied its properties. He was unable to crystallize it, but he observed the effect of various reagents, including acid and alkalis, on the solution and found it to turn red in acid, green in alkali.

Waksman (1919) described an organism which he called *Actinomyces violaceus-ruber*. He stated that this organism produced a soluble red-blue pigment which had indicator properties. His description was not as detailed as that of Müller,

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but his organism seemed to be very similar to Müller's, if not identical with it. Waksman himself later came to the conclusion that the two organisms were probably identical, and in the 5th edition of Bergey *Actinomyces violaceus-ruber* was, at his suggestion, considered synonymous with *Actinomyces coelicolor*. The available evidence, however, did not seem sufficient to settle this point satisfactorily either way. It is interesting to note in this connection that Kriss (1937) discussed in detail the variability of *Actinomyces coelicolor*. Then, in an entirely different section of his article, he referred to a culture of *Actinomyces violaceus-ruber* obtained from Waksman, giving the impression (although he did not actually say so) that he considered the two to be different organisms.

Conn (1921) was perhaps the first to call attention to the importance of the composition of the medium in studying pigment production by *Actinomycetes*. He observed that the same species would produce a different color on different media, and suggested that it might be due to the final hydrogen ion concentration. This work was confirmed by Tompel (1931) who found variation in color on different media but constancy under constant conditions. Further work along this line was done by Plotho (1940) who observed that the color produced by the various pigment-producing species of *Actinomyces* was generally darker in an alkaline medium than in an acid one. Furthermore, when the pigment was made acid, it was less soluble and precipitated on heating.

The chemical nature of these pigments has been discussed by Erikson, Oxford, and Robinson (1938), Frampton and Taylor (1938) and Kriss (1936), but without reaching very definite conclusions. The chief point at issue has been whether or not the pigments are anthocyanins like certain red-blue pigments of higher plants.

The attempts of the early workers to classify the *Actinomycetes* according to pigment production were all unsatisfactory. More recently Waksman (1940) has developed a method of classification by which the family is divided into five sub-groups according to the arrangement of the spore-producing bodies on dried agar plates. His method, however, is still not entirely satisfactory because the form of the spore-producing bodies is so variable and so hard to determine that individual investigators frequently disagree on the condition present in any one species. Kriss (1937) believes that the *Actinomycetes* can be classified if the classification is based upon many characteristics, if the morphological and physiological characteristics are broadly used, and if variability because of the outside environment is taken into consideration. He does not consider descriptions and classifications of any value which are based on single characteristics.

EXPERIMENTAL

Purpose

In view of the existing confusion, further work with this group of organisms seemed desirable. Accordingly, two pigment-producing strains of *Actinomyces* isolated from soil were selected for study. One of these produced a blue pigment on the synthetic agar used, the other a violet pigment. A comparison of the

cultural characteristics and of the chemical properties of the pigments of the two strains was undertaken with two objects in mind: (1) to determine whether or not the pigments produced by these two strains were identical, and (2) to determine whether either of them agreed with the description of Müller's *Actinomyces coelicolor* or with that of Waksman's *Actinomyces violaceus-ruber*.

Superficial appearance of organisms

For routine study the cultures were grown on a synthetic agar containing 0.085 per cent asparagin, 1 per cent glycerol and 0.1 per cent K_2HPO_4 . This is a modification of the medium described by Conn (1921) as giving fairly characteristic and vigorous growth with the majority of strains of *Actinomyces* used in his work. The two organisms used in this study were designated as V-1 and B-3. When grown on this medium, V-1 produced a leathery violet-colored surface growth with a white aerial mycelium and colored the medium a somewhat lighter violet. On the other hand, B-3 produced a rather moist blue surface growth with an abundance of white aerial mycelium and colored the medium a very deep blue. Thus, these two cultures were quite different in appearance and their pigments did not bear any superficial resemblance to one another. Furthermore, neither of them bore any marked resemblance to the culture obtained from Waksman as typical of his *Actinomyces violaceus-ruber*. On the same medium the last mentioned organism produced a reddish-violet surface growth with very scant white aerial mycelium, sometimes lacking entirely. The medium was colored a faint red or violet.

Cultural study

When a more detailed cultural study was undertaken, one of the first things noted was that the B-3 culture was contaminated, and probably had been from the beginning. The contaminating rod was not only easily overlooked, but proved rather difficult to eliminate. However, a pure culture was finally obtained, and it was interesting to note that this purified B-3, instead of producing a deep blue pigment, produced a violet pigment even lighter than that produced by V-1. This led to the suspicion that the two organisms might be identical after all. When NaOH was added to the medium upon which either culture had been grown, a deep blue color was produced similar to that observed in the contaminated culture of B-3. This would make it seem probable that the bacterial contaminant interfered in some way with the acid production by the *Actinomyces*, thus changing the pH and causing the pigment to appear in its alkaline phase instead of showing the intermediate violet hue.

Previous work (Conn and Conn, 1941) had shown that, when glucose was added to the glycerol-asparagin medium described above, the pigment produced by these cultures was an insoluble red instead of a soluble violet. Increasing amounts of glucose were used and the final hydrogen-ion concentrations of the cultures were determined. The results showed that increasing the amount of glucose caused an increase in the final hydrogen-ion concentration and a corre-

TABLE 1
Cultural studies

CULTURAL CHARACTER	V-1	B-3 (PURIFIED)	STREPTOTHEX COMBICOLOR (MÜLLER'S DATA)	ACTINOMYCES VIOLACEO-RUBER (WAKSMAN)	
				Observed	Waksman's data
Standard agar at 25°	Light blue White aer. myc.	Scant blue White aer. myc.	Good growth No aer. myc.	Medium blue White aer. myc.	Blue White aer. myc.
Standard agar at 37°	Very scant blue White aer. myc.	No pigment White aer. myc.	No pigment	No pigment White aer. myc.	Not reported
Gelatin at 18°	Liquefaction starting in 7 days	Liquefaction starting in 7 days	Liquefaction starting after 4-6 days	Very slight liquefac- tion in 14 days	Liquefaction slow to medium
Milk at 25°	No coagulation	No coagulation	No coagulation	No coagulation	Not reported
Milk at 37°	Rennet curd and pro- teolysis on 3rd day	Rennet curd and pro- teolysis on 3rd day	Rennet curd in 3 to 4 days	Rennet curd and pro- teolysis on 5th day	No coagulation; hy- drolysis and alka- line reaction in 15 days
Nitrate reduction	Positive	Positive	Positive	Positive	Not reported
Hemolysis of blood agar at 37°	Hemolysis on 4th day	Hemolysis on 4th day	Hemolyzed very rapidly	No hemolysis in 9 days	Strong hemolysis
Potato plug at 25°	Deep blue—7 days	Deep blue—7 days	Deep blue	Colorless	Blue—4-5 days
Fermentations	Glucose + Sucrose - Lactose + Mannitol -	Glucose + Sucrose + Lactose + Mannitol +	No acid from carbo- hydrates	Glucose + Sucrose - Lactose + Mannitol +	Not reported

sponding change in color from the soluble violet (alkaline phase) to the insoluble red (acid phase of the pigment).

After the B-3 culture was purified, cultural studies were made of these two experimental organisms and also of the culture of *Actinomyces violaceus-ruber* obtained from Waksman. The results of these studies are listed in table 1 together with the characteristics observed by Waksman (1919) for his organism and those given by Müller (1908) in his description of *Streptothrix coelicolor*.

From observation of table 1 it can be seen that the organisms V-1 and B-3 agree absolutely in all cultural characteristics tested except in the matter of acid production from carbohydrates and mannitol. B-3 produced acid from all carbohydrates used, but V-1 produced no acid from sucrose and mannitol. Furthermore, in all respects except this same matter of acid production the two experimental organisms agree with the description given by Müller for *Streptothrix coelicolor*. In regard to *Actinomyces violaceus-ruber* it will be noted that the characteristics observed for the organism do not agree with those reported in the literature in regard to coagulation of milk, hemolysis of blood agar, and production of pigment on potato plugs. This would make it appear probable that the strain of *Actinomyces violaceus-ruber* carried by Waksman in his stock cultures had become somewhat weakened by repeated transfers since it was originally described. Even the original description, however, disagrees with the other cultures studied since Waksman states that it shows good growth and abundant pigment production on standard agar, slow liquefaction of gelatin, and no coagulation of milk at 37°.

The matter of acid production from carbohydrates is rather hard to determine for these organisms without making time-consuming electrometric measurements. The indicator media ordinarily used for these tests are not satisfactory since the color of the indicator may well be masked by the pigment produced by the organism. Müller reported that there was no acid produced from carbohydrates when added in 1 per cent amounts to litmus ascites agar. This may mean that the color change of the litmus was masked by pigment production, or it may be that the medium used was not sufficiently favorable to permit acid production by the organism. In the present work, the carbohydrates tested were added to the synthetic agar described above in 2 per cent amounts and the production of acid was judged by observation of the indicator properties of the pigment. If the pigment produced was an insoluble red or light violet, acid was assumed to be present. On the other hand, if the pigment was a soluble blue-violet, it was concluded that no acid was produced. This method would seem to be fairly reliable. Nevertheless, the differences in acid production between V-1 and B-3 might well be due only to more active growth on the part of B-3.

If acid production from carbohydrates is disregarded, V-1 and B-3 appear to be identical with Müller's *Streptothrix coelicolor*, but not with Waksman's *Actinomyces violaceus-ruber*.

Extraction of pigments

In order to study the chemical properties of the pigments it was necessary first to extract them from the medium on which the organism had been grown. Nu-

merous laboratory solvents² were tried in an attempt to remove the pigment from an agar medium, but dioxane and NaOH were the only ones found to be effective. Both of these methods proved to be extremely slow, and required large amounts of solvent in proportion to the amounts of pigment obtained.

While searching for a better method, the discovery was made that the blue color produced on potato could be washed out readily with distilled water. Accordingly, the cultures were incubated on mashed potato for about four weeks at 25°C. At the end of this time the potato was colored a deep blue. The pigment was then extracted with distilled water, filtered through a Berkefeld filter, and sterilized. This method had several disadvantages: first, the extraction is very slow owing to the time required for filtering; secondly, the product obtained is not very pure; and third, it tends to decompose on standing, possibly because of the presence of organic matter from the potato. The pigment solution used by Müller in his studies of *Streptothrix coelicolor* was obtained by this method, although he admits that the solution was not pure.

Because of these disadvantages, further efforts were made to extract the pigment from agar cultures. The following method was finally developed: The culture was grown on glycerol-asparagin agar in large flasks, and incubated at 25°C., for two to four weeks, until the agar was colored a deep blue. The pigment was then extracted with very dilute NaOH and filtered. Fairly concentrated H₂SO₄ (about 10 N) was added to the deep blue solution resulting, causing it to turn red and precipitate. The precipitate was separated by centrifuging and dried.

The above method was satisfactory, but an even better method proved to be that described by Conn and Botcher (1942), in which the organism was grown on cotton saturated with the nutrient solution used in making the agar. The pigment could be easily and quickly extracted from the cotton by squeezing it. The solution was then precipitated and centrifuged as before. The advantages of this method are that the organism grows faster on cotton than it does on agar, and the extraction is easier and quicker.

This method of extracting the pigments yielded a product that was realized to be far from pure. After various unsuccessful attempts to find a reagent which would precipitate them in crystalline form and therefore permit some hopes of actual purification, the effort to purify them was temporarily abandoned. The products obtained by precipitation from the extract of a culture growing on a synthetic medium were much purer than the extracts themselves (in that the substances soluble in dilute H₂SO₄ had been largely eliminated) and were very much purer than the extracts of organic media (*e.g.*, potato). In fact it was found that these precipitates were sufficiently pure to yield more valuable data concerning the solubilities of the pigments than could be obtained from the cultures before such treatment; and solutions made from them could be compared with one another by spectrophotometric analysis and thus supply some information as to the identity of the pigments present. Attention is specially called to

² See list in table 2. The following additional solvents were included: Dilute HCl, CCl₄, CH₂Cl₂, pyridine.

this method of studying pigment-producing Actinomycetes. A distinct advantage of the spectrophotometer in studying such colored substances is that it furnishes much information concerning their nature without actual isolation of the pigments themselves.

Solubilities of pigments

The pigments of the two experimental organisms agreed with those of both Müller's (1908) organism and that of Waksman (1919) in that all were blue or green in alkali and red in acid. To determine whether or not the pigments are actually identical, a further study was made of their solubilities, using the two experimental organisms and the culture obtained from Waksman. For this purpose the pigments were extracted as described above, and small amounts of the washed and dried extract were added to the different reagents. The results are recorded in table 2. Also, for comparison are recorded as much about the solubilities of Müller's pigment as could be determined from his description. His results, however, are not strictly comparable since he used an extract obtained by washing the pigment out of potato with water and evaporating. By his own admission this extract was not pure.

From a study of the table, it can be seen that the pigment produced by the V-1 organism behaves in very much the same way as that produced by B-3. The only conspicuous differences are that the V-1 pigment is more soluble in amyl alcohol and the B-3 pigment is more soluble in phenol. Then the pigment from B-3 is very slightly soluble in benzene and xylene whereas that from V-1 is insoluble. The pigment from Waksman's organism on the other hand behaves very differently. It is generally less soluble in all reagents, being readily soluble only in NaOH. In acid solution it is insoluble; in alkali it is soluble but turns a light blue instead of the deep blue or green produced by the other pigments. In Waksman's description of the organism, he does not discuss these properties of the pigment so that it is impossible to tell whether or not the pigment has changed in nature since the culture was first isolated. From the data in the table, certain conclusions may be drawn. First, the pigments produced by V-1 and B-3 are probably identical, since it is hard to imagine that two different pigments would be so much alike in their solubilities. Second, the pigment isolated from Waksman's culture of *Actinomyces violaceus-ruber* is quite different from either of these. Third, judging from the reactions in acid and alkali, the pigment isolated from the experimental organisms resembles the pigment from Müller's organism much more closely than that from Waksman's.

Certain investigations were made to obtain some idea as to what might be the chemical nature of the pigment. The results were inconclusive, however, and as they add nothing to what is already in the literature, they are not reported here.

Spectrophotometric determinations

To obtain further evidence as to whether or not the pigments produced by these various cultures are identical, spectrophotometric studies of the pigments

TABLE 2
Solubility of pigments

SOLVENT	MÜLLER'S DATA CONCERNING STREPTOTHRIN COELICOLOR	PIGMENTS EXTRACTED FROM		
		V-1	B-3	Waksman's <i>Actinomyces violaceus-ruber</i>
Water.....	soluble—blue	v. sl. soluble—faint pink	sl. soluble—pink	insoluble
N/10 H ₂ SO ₄	soluble—red	v. sl. soluble—faint pink	sl. soluble—pink	insoluble
10N H ₂ SO ₄	soluble—red	soluble—red	soluble—red	insoluble
N/10 NaOH.....	soluble—green	very soluble—dark blue	very soluble—dark blue	soluble—light cloudy blue
N/1 NaOH.....	soluble—green	very soluble—dark blue green after 24 hrs.	very soluble—dark blue green after 24 hrs.	soluble—pale blue
Ethyl alcohol (absolute).....	insoluble	soluble—red	soluble—red	v. sl. soluble—faint pink
Methyl alcohol.....		very soluble—deep red	very soluble—deep red	sl. soluble—pink
Amyl alcohol.....		soluble—red	sl. soluble—pink	v. sl. soluble—faint pink
Benzene.....	insoluble	insoluble	v. sl. soluble—faint pink	insoluble
Xylene.....	insoluble	insoluble	v. sl. soluble—faint pink	insoluble
Phenol.....		sl. soluble—pink	soluble—red	insoluble
Dioxane.....		very soluble—deep red	very soluble—deep red	partially soluble—red
Carbon disulfide.....		insoluble	insoluble	insoluble
Ethyl ether.....	insoluble	sl. soluble—pink	v. sl. soluble—faint pink	v. sl. soluble—faint pink
Acetone.....		very soluble—deep red	very soluble—deep red	sl. soluble—pink
Chloroform.....	insoluble	insoluble	insoluble	insoluble
Petroleum ether.....		insoluble	insoluble	insoluble
Formaldehyde.....		very soluble—deep red	very soluble—deep red	sl. soluble—pink
Ethylene chloride.....		insoluble	insoluble	insoluble
Acetaldehyde.....		soluble—red	soluble—red	v. sl. soluble—faint pink

were made. The first studies were made on the pigments extracted from the potato cultures. These results were not satisfactory, however, because the solutions were so unstable that curves made two hours apart from the same solution were not alike. Subsequent studies were made using the aqueous extracts, both partially purified (by precipitation with H_2SO_4) and unpurified.

Figure 1 was made using the results obtained from the partially purified NaOH extract of B-3. Curve 1 was prepared from the unbuffered solution which had a pH of 10.73. The solution used for Curve 2 was buffered to pH 6.3 and that for

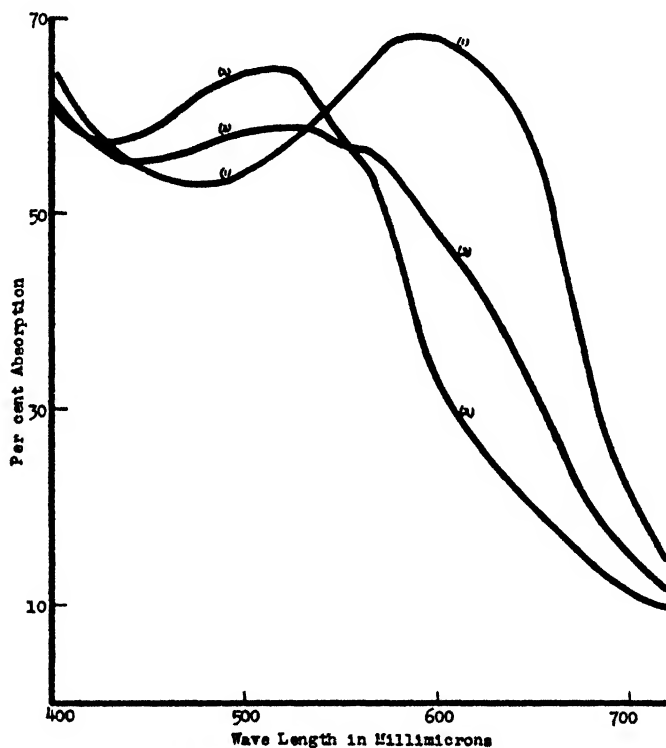


FIG. 1. A COMPARISON OF THE ABSORPTION SPECTRA OF AQUEOUS SOLUTIONS OF PIGMENT FROM B-3 AT DIFFERENT HYDROGEN-ION CONCENTRATIONS

Curve 1, unbuffered; pH 10.73. Curve 2, buffered to pH 6.3. Curve 3, buffered to pH 7.9.

Curve 3 to pH 7.9. These curves also show the shift in wave length of the maxima from the acid to the alkaline solutions, with the maximum at 520 $m\mu$ at pH 6.3; 530 $m\mu$ at pH 7.9, with a secondary maximum at 560; and 590 $m\mu$ at pH 10.73. Figure 2 shows similar curves made from the purified NaOH extract of V-1. These curves are not absolutely identical with those of figure 1, but they have their maxima at absolutely the same wave lengths, even to the secondary maximum at 560 observed at pH 8.0.

The curves in figure 3 were made from aqueous unbuffered and unpurified solutions of the pigments of B-3 and V-1 that had been poured off from the base

of the flasks in which the cultures had been grown. These curves do not show a great deal in themselves, but are included for comparison with the data given by Müller (1908) concerning the spectrum of a similar extract of his organism. He states that the absorption was strongest between the D line ($589.5\text{ m}\mu$) and the beginning of the pure green part of the spectrum (about $550\text{ m}\mu$) becoming gradually less on each side of this region until the C line ($656.3\text{ m}\mu$) and the E line ($526.9\text{ m}\mu$) were reached. Since his determinations were made from an unpurified solution, the results should be more nearly comparable with the curves shown in figure 3 than those of figure 1 and figure 2. The region where he observed the

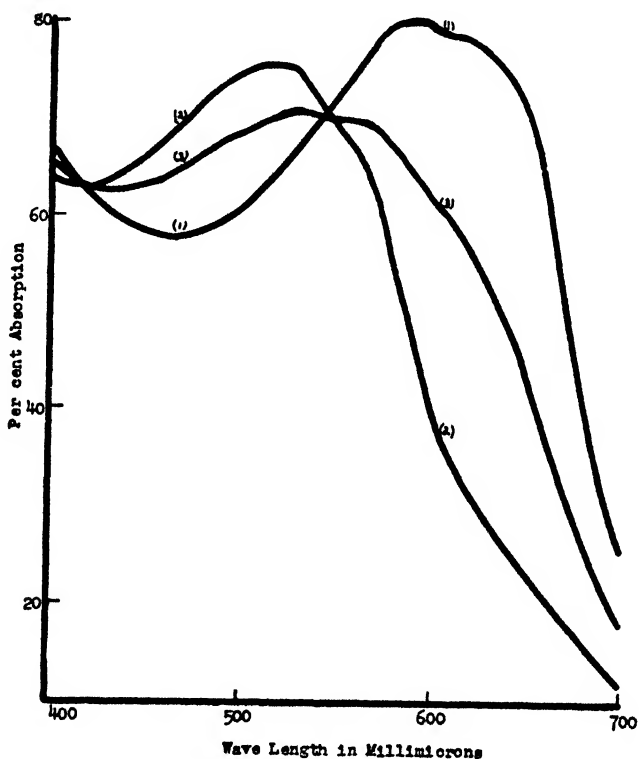


FIG. 2. A COMPARISON OF THE ABSORPTION SPECTRA OF AQUEOUS SOLUTIONS OF PIGMENT FROM V-1 AT DIFFERENT HYDROGEN-ION CONCENTRATIONS

Curve 1, unbuffered; pH 10.58. Curve 2, buffered to pH 6.3. Curve 3, buffered to pH 8.0.

most absorption is indicated by the block drawn at the base of figure 3. The limits of absorption are indicated by the lines drawn at 525 and $660\text{ m}\mu$.

Figure 4 shows curves made from the purified NaOH extract of Waksman's culture of *Actinomyces violaceus-ruber*. The solution used in Curve 1 was buffered to pH 6.3, that in Curve 2 to pH 7.2, Curve 3 to pH 7.7, and Curve 4 to pH 9.2. These curves are quite different in appearance from those shown in figure 1 and figure 2. The absorption maxima occur at approximately the same wave lengths, but are much less pronounced. The most alkaline solution

(Curve 4) has its maximum around 580 $m\mu$ which is about the same as the maxima for the pigments of V-1 and B-3. However, the more acid solutions have their maxima a little further to the left than do the corresponding ones for the other pigments. This might indicate that this pigment would change from blue to red at a higher pH than the other pigments. This observation is in accord with Waksman's statement (1919) that the pigment changes color at pH 7.6.

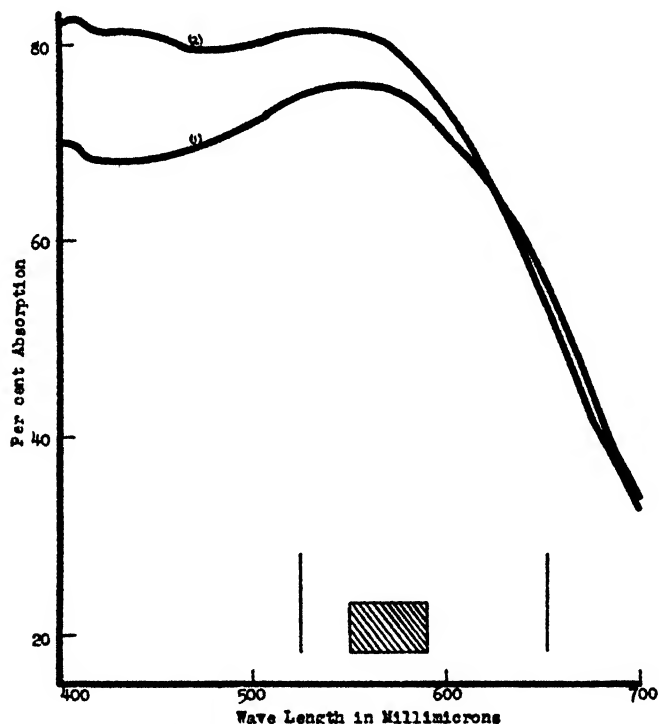


FIG. 3. A COMPARISON OF THE ABSORPTION SPECTRA OF UNPURIFIED AQUEOUS SOLUTIONS B-3 AND V-1 PIGMENTS WITH THE ABSORPTION DATA GIVEN BY MÜLLER FOR *STREPTOTHRIX COELICOLOR*

Curve 1, B-3 pigment at pH 7.35. Curve 2, V-1 pigment at pH 7.15.

The block at the bottom of the figure indicates the region where Müller observed the most absorption. The limits of absorption are indicated by the lines on either side.

A study of these curves shows beyond a doubt that there is no difference spectrophotometrically between the pigments produced by the organisms V-1 and B-3. In regard to the pigment from Waksman's *Actinomyces violaceus-ruber*, one of two things is indicated. Either this pigment is a quite different substance from that produced by the other organisms, or else the substance is a mixture of several compounds including a small amount of the same pigment which is found in the other cultures. This would be in agreement with Waksman's conclusion that the coloring matter produced by *Actinomyces violaceus-ruber* is a mixture of several pigments. No definite conclusions can be drawn con-

cerning Müller's organism from the absorption data which he gives, especially since he does not mention the pH of his solution; but the maximum which he gives is closer to that observed for the pigments of V-1 and B-3 than is that of Waksman's organism. The only curve for *Actinomyces violaceus-ruber* which shows a maximum between 550 and 590 $m\mu$ is the one made at pH 9.2, and it does not seem probable that Müller's solution was as alkaline as that.

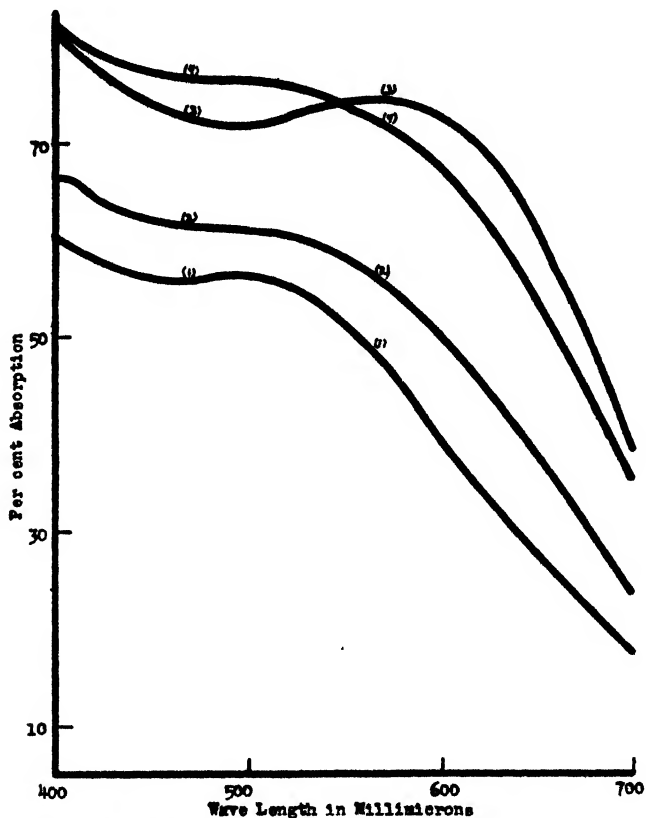


FIG. 4. A COMPARISON OF THE ABSORPTION SPECTRA OF AQUEOUS SOLUTIONS OF PIGMENT FROM *ACTINOMYCES VIOLACEUS-RUBER* BUFFERED TO DIFFERENT HYDROGEN-ION CONCENTRATIONS

Curve 1, buffered to pH 6.3. Curve 2, buffered to pH 7.2. Curve 3, buffered to pH 7.7. Curve 4, buffered to pH 9.2.

Comparison with litmus

Beijerinck (1914) mentions *Actinomyces coelicolor* as producing a pigment similar to that of Schröter and Cohn's "litmus micrococcus," *Micrococcus cyaneus* Cohn, and suggests that one of these organisms may have some connection with the commercial preparation of litmus. Beijerinck makes no mention of having made spectrophotometric studies. It seemed worth while, therefore, to make a comparison of litmus with the pigment of *Actinomyces coelicolor* by means of the spectrophotometric methods now available. For this purpose a solution was

made of a commercial sample of litmus purchased several years ago; also a solution of azolitmin, believed to have been imported before the war, was obtained from a commercial house in this country.

The absorption spectra of the pigments from culture B-3, of litmus, and of azolitmin were obtained at three different H-ion concentrations by mixing them with phosphate buffer solutions having the reactions of pH 6.2 and 8.0 respectively, as well as with a strongly alkaline solution (about pH 11-12). The absorption spectra in the alkaline solution are given in figure 5. The similarity

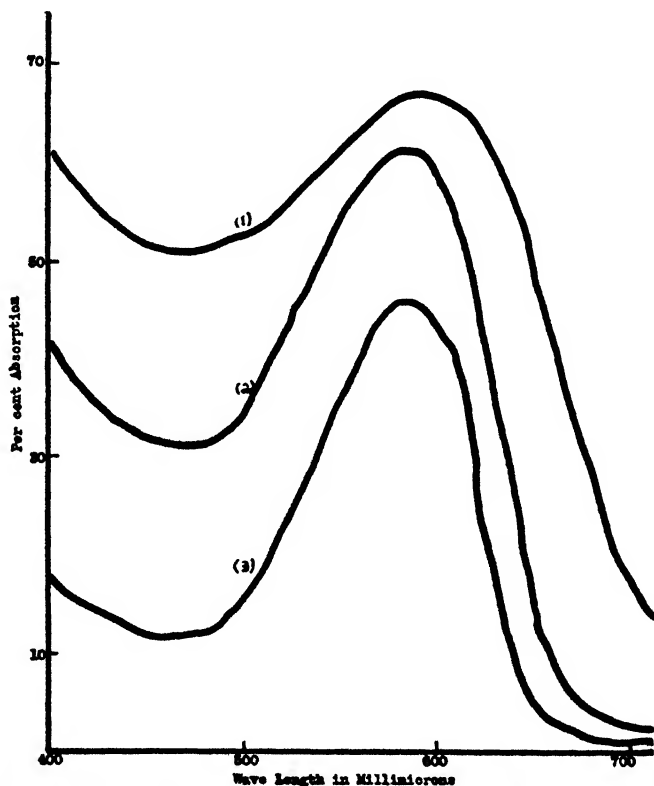


FIG. 5. A COMPARISON OF THE ABSORPTION SPECTRUM OF PIGMENT FROM B-3 WITH THOSE OF AZOLITMIN AND LITMUS; ALL IN ALKALINE AQUEOUS SOLUTION (pH 11 to 12)
Curve 1, actinomyces pigment. Curve 2, azolitmin. Curve 3, litmus.

of these curves is very striking both in their general shape and in the position of the maximum (close to 580 $m\mu$).

Under acid conditions (pH 6.2), as will be seen from figure 6, a quite different type of curve was obtained with its maximum at about 500 $m\mu$. (This is merely another way of stating that the solutions were then red instead of blue.) At this reaction, however, the curve for litmus proved distinctly different from those for azolitmin and for the pigment, in that it had a secondary maximum at about 580 $m\mu$. Now azolitmin is generally regarded as the primary active constituent of litmus, although other colored compounds are known to be present. The

curve for litmus at pH 6.2 indicates that even under conditions as acid as this some substance is present which absorbs light of about 580 m μ wave length, although the blue color is not evident to the eye.

At a reaction of pH 8.0 (fig. 7) the differences between the *Actinomyces* pigment and either of the other colored substances became more evident. The pigment showed its maximum at about 530 m μ while that of litmus and of azolitmin was around 580 m μ . This indicates that the color of this pigment at this reaction

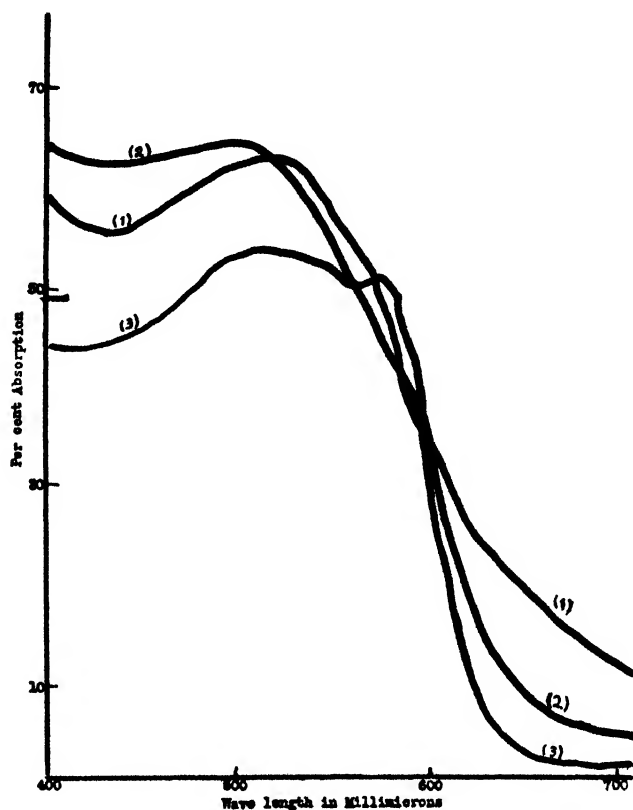


FIG. 6. A COMPARISON OF THE ABSORPTION SPECTRUM OF THE PIGMENT FROM B-3 WITH THOSE OF AZOLITMIN AND LITMUS; ALL IN AQUEOUS SOLUTION BUFFERED TO pH 6.2

Curve 1, actinomyces pigment. Curve 2, azolitmin. Curve 3, litmus.

is somewhat redder than azolitmin (which is purplish in hue) and very much redder than litmus (which is blue). In other words the pH-value (dissociation constant) for the *Actinomyces* pigment is higher than that of azolitmin or litmus; in fact it is too high to allow it to be used as a substitute for litmus in certain indicator media (e.g., milk) used by the bacteriologist. This would indicate that although the pigment produced by this organism is very similar to azolitmin it is not identical with it, and it would hardly be suitable to nick-name this culture "the litmus *Actinomyces*".

By comparing these curves with those for the pigment from *Actinomyces violaceus-ruber* at various reactions (fig. 4) it will be seen that the latter pigment is even less like azolitmin.

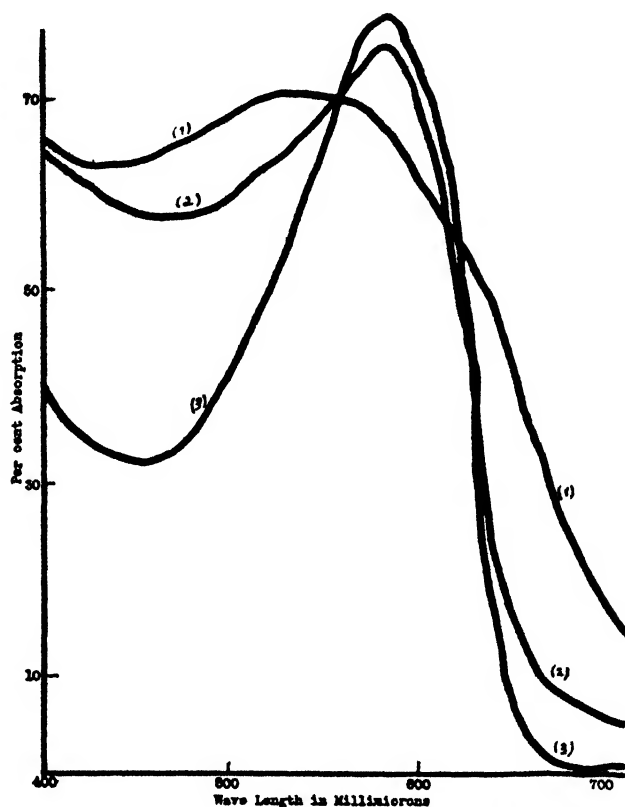


FIG. 7. A COMPARISON OF THE ABSORPTION SPECTRUM OF THE ACTINOMYCES PIGMENT (FROM B-3) WITH THOSE OF AZOLITMIN AND LITMUS; ALL IN AQUEOUS SOLUTION BUFFERED TO pH 8.0

Curve 1, actinomycetes pigment. Curve 2, azolitmin. Curve 3, litmus.

DISCUSSION

These experiments have not completely clarified the confusion existing in the literature in regard to these pigments, but they have shown several reasons for this confusion. The difference in appearance of the B-3 culture before and after purification is interesting to note in this connection. If a contaminant, as hard to detect and to remove as this one was, can cause two almost identical cultures to look and to act so differently, it is no wonder that investigators have observed the same organism to produce different colors at different times even when grown under identical conditions. Further confusion has probably been caused by the indicator properties of the pigments. Many investigators have reported results on a "synthetic medium" without giving the exact composition of the medium. It can be readily seen how a slight variation in the composition of the

medium or in the vigor of the culture might alter the final hydrogen ion concentration of the organism, and thus cause the pigment to appear a different color. The temperature of incubation also has some effect. The organisms studied here will produce pigment well at 25° and 30°C., but not at 37°, even though they will grow well at the higher temperature.

This work has shown very clearly the similarity between the organisms designated as V-1 and B-3, which were thought at first to be different species. Culturally they are almost identical, and their pigments, studied both chemically and spectrophotometrically, appear to be the same compounds. During the entire course of investigation, no evidence was found to indicate that any of the organisms (except possibly Waksman's *Actinomyces violaceus-ruber*) produced more than one pigment.

In comparing these two cultures with the organisms described in the literature, it is evident that, in their cultural characters, they agree very closely with Müller's description of *Streptothrix coelicolor*, but are somewhat different from Waksman's *Actinomyces violaceus-ruber*. This distinction is further supported by chemical and spectrophotometric studies of their respective pigments. In view of this evidence, it appears probable that there are at least two species of *Actinomyces* which produce a pigment changing from blue to red that acts as a hydrogen-ion indicator. Because of the superficial resemblance of their pigments they appear to be quite closely related, but a more careful examination of their cultural characteristics and of the chemistry of their pigments indicates them to be distinct species. This contention is further supported by the work of Kriss (1937). His *Actinomyces coelicolor* is apparently the same organism as Müller's *Streptothrix coelicolor* and the two cultures studied here. In another part of his paper, however, he makes a reference to a culture of *Actinomyces violaceus-ruber* obtained from Waksman, and implies that he considers the latter a different organism.

This work shows that the members of the genus *Actinomyces* cannot be satisfactorily classified from superficial appearance alone. It is necessary, in accordance with the conclusions of Kriss (1937) to make careful cultural studies under controlled conditions. Furthermore, it would be advisable to supplement these studies with simple chemical tests of their pigments, especially as to their solubilities and their reactions with acids and alkalis, while spectrophotometric studies of partially purified preparations of these pigments are regarded as of special value.

CONCLUSIONS

From the above experiments, the following conclusions may be drawn:

1. Chemical and spectrophotometric studies show that identical pigments are produced by two strains of *Actinomyces* isolated from soil and thought at first to be separate species because of differences in color observed on superficial examination. This pigment is very similar to (but not identical with) azolitmin, the best known constituent of litmus.

2. The two strains are so much alike in their cultural characters that they may be considered the same species.

3. This species is the same one described by Müller as *Streptothrix coelicolor* and by Kriss as *Actinomyces coelicolor*. It is listed in the 5th edition of Bergey's Manual as *Actinomyces coelicolor*.

4. Waksman's *Actinomyces violaceus-ruber* is quite different culturally and in the chemistry of its pigment. Therefore, the name should probably be retained as that of a species distinct from *Actinomyces coelicolor*.

5. The method of comparing such cultures by spectrophotometric study of partially purified pigments proves sufficiently promising so that it is recommended for application to other pigment-producing Actinomycetes.

ACKNOWLEDGMENT

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Further appreciation is expressed to Mrs. A. P. Bradshaw of the Biological Stain Commission and Dr. B. A. Brice of the Eastern Regional Research Laboratory at Philadelphia for their assistance in making the spectrophotometric determinations; also to Dr. O. D. Frampton of the Biochemistry Department of Cornell for his helpful suggestions in regard to the chemical phases of the investigation.

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THE EFFECT OF GERMICIDES ON THE VIABILITY AND ON THE RESPIRATORY ENZYME ACTIVITY OF GONOCOCCUS¹

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The relationship between enzyme activity and destruction of bacteria by disinfectants has been the subject of numerous investigations in recent years. In 1927, Quastel and Wooldridge studied the effect of chemical and physical agents on various dehydrogenases of *Escherichia coli*. In several experiments, the treated organisms were subcultured to compare the viability of the organisms with their enzyme activity. When the dehydrogenases were completely inhibited (particularly those dehydrogenating the sugars), the organisms were found to be dead or greatly reduced in number. Casman and Rettger (1933), working with the effect of different temperatures on several species of the genus *Bacillus*, as well as some strict thermophiles, pointed out that the succinic dehydrogenase system of most of the organisms appeared to be distinctly inhibited at the maximum temperature of growth. Paraphenylenediamine oxidase and catalase activities varied considerably in this respect. Qualitative methods were employed to detect the enzyme activities. Similar studies were also made by Edwards and Rettger (1937) as well as by Wedberg and Rettger (1941) with a wider variety of organisms; essentially the same methods as those of Casman and Rettger were used.

Yudkin (1937) demonstrated that silver sulphate was lethal to suspensions of *E. coli* in concentrations much lower than those inhibiting the dehydrogenases (glucose, succinic, lactic and formic), hydrogenase and formic hydrogenlyase. Sykes (1939) studied the effect of alcohols and phenol derivatives on succinic dehydrogenase of *E. coli*. It was found that at concentrations of the germicides equal to those killing the organisms, the activity of the enzyme was considerably or completely inhibited.

Rahn and Barnes (1933) reported on the different criteria of death of yeast cells. When the organisms were subjected to the action of heat or mercuric chloride, the rate of loss of reproduction was about twice as rapid as the loss of fermentation, about three times that of the loss of semipermeability, and about forty times as large as the rate of coagulation of the protoplasm. Rahn and Schroeder (1941) investigated the problem of enzyme inactivation as the cause of death of bacteria. Contrary to the work of Edwards and Rettger (1937), these workers found that when half of the catalase and succinic dehydrogenase activities of *Bacillus cereus* were inhibited at 46° and 50°C., more than 99.9 per

¹ This paper covers in part a dissertation submitted to the Graduate School of New York University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

cent of the cells were found to be dead. The effect of solutions of mercuric bichloride, phenol (glycerinated) and trypanflavine on formic and lactic dehydrogenase of *Proteus* OX₁₉ was studied by Braun and Vásárhelyi (1940). It was found that the dehydrogenases were inactivated by concentrations of the compounds which lie far below those necessary to kill the organisms.

Investigations on the effect of therapeutically active drugs employed in the treatment of gonococcal infections on the viability of *Neisseria gonorrhoeae* are numerous (Swartz and Davis, 1921; Thomas and Bayne-Jones, 1936; Thomas, 1939). However, the effect of these chemical agents on the respiratory enzymes of this pathogen is virtually unknown, although the gonococcus has been shown by Barron and Miller (1932) to have a relatively simple respiratory enzyme system. The purpose of the present investigation is to present a study of the relationship between the lethal concentration of drugs and those concentrations necessary to inactivate certain of the respiratory enzymes of the gonococcus. The degree of inhibition of the enzymes at the lethal concentration level of the gonococci was also studied.

EXPERIMENTAL PROCEDURES

Test organism. The strain of *Neisseria gonorrhoeae* used in this investigation was isolated from the cervix of a patient with a clinical diagnosis of chronic gonococcal endocervicitis, according to the method described by Carpenter (1937). The culture is designated No. 1111. Its cultural, microscopical, tinctorial, and fermentative characteristics were found to be typical of the gonococcus.

Medium used. The organisms were cultivated routinely on beef-heart agar modified from the original method of Torrey and Buckell (1922) (medium B). The medium was enriched by the addition of 20 per cent egg digest prepared according to the method of Price (1935). Bacto proteose peptone No. 3 was substituted for Difco peptone. The agar concentration of the preparation was two per cent and the final pH was 7.2.

Suspending and diluting fluid. Since solutions of silver salts cannot be prepared in physiological sodium chloride and since distilled water cannot be used as a suspending fluid for gonococci, it was found necessary to use another suitable isotonic salt solution. This consisted of a 0.15 M sodium acetate solution ($\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$) in distilled water adjusted to pH 6.6 with acetic acid. Suspensions of organisms, as well as dilutions of the drugs, were always prepared with sterile acetate solution. All solutions and reagents were prepared in redistilled water from an all-glass still.

Germicides employed. The drugs tested in this investigation were those which have been in common use for the treatment of gonococcal infections. They were: silver nitrate, protargol ("strong" silver protein compound), neo-silvol (colloidal silver iodide-protein compound), silver nucleinate ("mild" silver protein compound), argyrol ("mild" silver protein compound), potassium permanganate, merthiolate² (sodium ethyl mercuri thiosalicylate), and sulfanilamide. Stock

² Merthiolate powder was kindly donated by Eli Lilly and Company, Indianapolis, Indiana.

solutions of the drugs were prepared in doubly distilled water in concentrations up to 20 gm. per 100 ml. of solution. These stock solutions were found to be sterile with the exception of sulfanilamide. The sulfanilamide stock solutions (1.5 gm. per 100 ml. solution) were sterilized separately by each of three methods: (a) filtration through a Seitz filter, (b) autoclaving at 15 lb. for 20 minutes, (c) heating in the Arnold sterilizer at 100°C. for one hour on each of three successive days. The results obtained by any of the aforementioned methods were found to be identical. The serial dilutions from the stock solutions of the drugs were always prepared in sterile acetate solution.

General procedure followed for conducting germicidal and enzyme inhibition tests. The centrifuge method of Davis and Swartz (1920) for testing the effect of germicides on suspensions of gonococci was employed, with certain modifications. The suspensions of organisms prepared for the tests were standardized with the use of a photoelectric colorimeter to a turbidity equivalent to the No. 5 barium sulfate standard of McFarland (1907). This standard was prepared by adding 5 ml. of 0.048 M BaCl_2 to 95 ml. of 0.1 M H_2SO_4 . The total count of this suspension was approximately three billion gonococci per ml. as determined in a Petroff-Hausser bacteria counter. From time to time in the course of this work, the standardized suspensions of organisms were checked by making total nitrogen determinations according to the method of Koch and McMeekin (1924). Values from 0.103 to 0.118 mg. total nitrogen per ml. of standardized suspension were obtained.

Kolle flasks were inoculated with a suspension prepared from 24-hour slants of gonococci. The flasks were incubated for a period of 48 hours in a moist incubator at 35° to 36°C. The growth was then suspended in sterile acetate solution, centrifuged for 20 minutes at approximately 3,700 r.p.m. and standardized in the photoelectric colorimeter. In the meantime, serial dilutions of the stock solutions of the drugs were prepared in sterile acetate solution in twice the final concentration desired. The germicide dilutions and the suspensions of organisms were used as soon as they were ready. Equal quantities of the drug dilutions and the gonococcus suspension were mixed and placed in a constant temperature water bath at 37°C. for exactly 20 minutes. The treated organisms were then sedimented in an angle type centrifuge for exactly 2.5 minutes at approximately 6,000 r.p.m. After the second washing, the organisms were finally resuspended in sterile acetate solution and restandardized to a turbidity equal to the No. 5 barium sulfate tube. The drug-treated organisms were tested for viability and enzyme activity. The enzyme actions investigated were: (a) lactic dehydrogenase activity, (b) glyceric dehydrogenase activity, (c) catalase activity, (d) peroxidase activity, and (e) indophenol oxidase activity.

The general procedure described was always followed for approximately the same length of time in order to make the various tests comparable. The viability and enzyme inhibition tests were first performed on organisms with ten-fold dilutions of the stock solutions of the drugs. Once the endpoint zone was determined, intermediate dilutions of the chemical agents were tested. In the case of the viability tests, an untreated organism control was included. In the

enzyme inhibition tests, a blank without organisms, an untreated organism control, and a control containing organisms heated at 100°C. for a period of 15 minutes were included with each group of tests performed at one time.

The endpoint selected for the germicidal tests was the highest dilution of the chemical agents which completely inhibited the growth of the treated gonococcal suspensions as determined by subculture. The endpoint selected for the enzyme inhibition tests was the highest dilution of the drugs necessary to inhibit enzyme activity completely under the conditions specified.

Germicidal test. One ml. of the standardized drug-treated suspension was inoculated into each of 10 ml. quantities of 20 per cent hydrocele Torrey broth and 5 per cent egg-digest Torrey broth. The broth cultures were incubated in a moist chamber at 35° to 36°C. for a period of seven days. Subcultures from these tubes were made immediately and after 48, 96, and 168 hours' incubation on Douglas' "chocolate" agar plates (Carpenter, 1937). The inoculated plates were incubated at 35° to 36°C. for 48 hours in an atmosphere of 10 per cent carbon dioxide.

Dehydrogenase tests. The activity of lactic and glyceric dehydrogenases was studied by the Thunberg methylene-blue technique (Thunberg, 1917-18; 1930). In preliminary tests, it was found that a solution of proteose peptone No. 3 (2 per cent) and NaCl (0.5 per cent) in distilled water may be advantageously employed to accelerate enzymatic activity. Controls showed that organisms in the presence of this solution did not reduce methylene-blue when lactate and glycerate were omitted in the test. The tubes were incubated in a constant temperature water bath, and observations were made for a period of 120 minutes. The time required to produce 90 per cent leucomethylene blue, or the percentage reduction of the dye at the end of the experimental period, was recorded. The standards were prepared as follows: to Thunberg-type tubes were added one ml. of standard suspension of organisms, varying concentrations of stock solution of methylene-blue, and buffer to make a final volume of 3 ml. The amount of reduction in the tests was estimated by comparison in a colorimeter block before a daylight bulb.

Quantitative catalase test. The catalase determinations were carried out with the drug-treated organisms according to the method of Kirchner and Nagell (1926). However, the total volume of the reaction mixture tested was smaller than that used by these investigators. The results were recorded as the quantity of standard potassium permanganate consumed in titrating the residual hydrogen peroxide.

Quantitative peroxidase test. The pyrogallol peroxidase test was also performed according to the method of Kirchner and Nagell (1926). As in the case of the catalase test, a smaller volume of the reaction mixture was also tested. The results were recorded as the quantity of purpurogallin formed.

Quantitative indophenol oxidase test. The oxidase test was developed after a number of preliminary tests were conducted to determine the optimal conditions for indophenol oxidase activity of gonococcus. Redistilled water from an all-glass still was particularly necessary for this determination since Wert-

heimer (1926) found that traces of metal impurities, such as copper and iron, accelerate the autoxidation of the Nadi reagent in air. The reagents were freshly prepared as follows: 0.144 per cent α -naphthol in 50 per cent ethyl alcohol; 0.108 per cent dimethyl-p-phenylenediamine HCl³ in distilled water; 0.044 per cent sodium carbonate (anhydrous) in distilled water.

The conditions for the test will be found in the footnote to table 8. The final pH of the test mixture was 6.8. The test was conducted in a constant temperature water bath at 37°C. for a period of exactly 15 minutes, after which the reaction was arrested by the addition of a 2 per cent solution of unneutralized KCN. The dyestuff formed was extracted with a mixture consisting of equal parts of ethyl alcohol and chloroform. The amount of extracted dye was determined in a Duboscq type colorimeter using alpha-naphthol blue standards. Alpha-naphthol blue (also called indophenol blue and p-dimethylaminophenyl- α -naphthoquinone-imine) was prepared by the interaction of α -naphthol with dimethyl-p-phenylenediamine HCl (Rowe, 1924) using ferric chloride to accelerate the reaction (Guthrie, 1931). The dyestuff was purified by three precipitations from ethyl alcohol, dried *in vacuo*, and stored in an amber glass-stoppered bottle. The values obtained in the tests were recorded as the quantity of α -naphthol blue formed.

RESULTS

The germicidal and enzyme inhibition endpoints recorded in the tables represent averages of at least three separate experiments. The results obtained with the silver protein compounds were expressed as molar concentrations of silver. Silver determinations of these silver protein drugs were made according to the permanganate sulphuric-acid oxidation method of F. Lehmann (Hagers Handbuch, 1938).

Lethal effect of germicides on gonococci. From the results shown in table 1, it is seen that silver nitrate and merthiolate were the most active lethal compounds, whereas sulfanilamide was not lethal for the gonococcus under the conditions of the test.

In vitro effect of sulfanilamide on gonococcus. Tests were performed to determine whether the strain of gonococcus tested in this investigation was either resistant or sensitive to the action of sulfanilamide. Sulfanilamide broth tubes were inoculated with the standard suspension of organisms as well as graded dilutions; namely, 1-10 and 1-100 dilution of the standard suspension. This was done because the *in vitro* effect of sulfanilamide has been found to be dependent upon the size of the inoculum (e.g. Weld and Mitchell, 1939). The results are shown in table 2. Over a period of 20 minutes, sulfanilamide in a concentration of 1-133 (4.36×10^{-3} M) apparently did not affect the viability of the gonococcus. However, death of the organisms occurred at least after 24 hours' exposure to the drug.

Inhibition of gonococcal lactic dehydrogenase. It is seen from table 3 that silver

³ A light colored product should be used so that the value of the blank remains low.

nitrate, potassium permanganate, and protargol were the most potent inhibitors of gonococcal lactic dehydrogenase activity among the substances tested.

Inhibition of gonococcal glyceric dehydrogenase. Table 4 shows that potassium permanganate, protargol, and silver nitrate were the most potent inhibitors

TABLE 1
*Minimal concentration of germicides lethal for the gonococcus**

COMPOUND	MIN. LETHAL CONCENTRATION (MOLAR)
Silver nitrate.	7.35×10^{-6}
Protargol	3.78×10^{-6} (Ag)
Neo-silvol.	1.63×10^{-6} (Ag)
Silver nucleinate	2.22×10^{-6} (Ag)
Argyrol	3.71×10^{-6} (Ag)
Potassium permanganate.	6.32×10^{-6}
Merthiolate	2.73×10^{-6}
Sulfanilamide.	Not lethal at 4.36×10^{-1}

* The standardized drug-treated suspensions were inoculated into egg-digest and hydrocele broth tubes. Exactly 0.2 ml. of the inoculated broths was plated on the surface of Douglas' chocolate agar plates at stated intervals of time. The inoculated broths and plates were incubated at 35° to 36°C. The broth tubes were examined for turbidity and the number of colonies appearing on the plates were enumerated and recorded.

TABLE 2
*The in vitro effect of sulfanilamide on the viability of the gonococcus**

TIME OF SUBCULTURE	STANDARD SUSPENSION (UNDILUTED)		STANDARD SUSPENSION (DILUTED 1-10)		STANDARD SUSPENSION (DILUTED 1-100)	
	Control†	Drug‡	Control†	Drug‡	Control†	Drug‡
20 minutes	++++	++++	++++	++++	++	++
24 hours	++++	0	++++	0	++++	0
48 hours	++++	0	++++	0	++++	0
168 hours.	++++	0	++++	0	++++	0

* One ml. of organisms was inoculated into 10 ml. of broth with and without sulfanilamide, and incubated under aerobic conditions in a moist incubator. At the intervals stated, 0.2 ml. of the inoculated media was placed over the surface of Douglas' chocolate agar plates. The plates were incubated in an atmosphere of 10 per cent CO₂ at 35° to 36°C.

† Torrey "B" broth enriched with 20 per cent sterile hydrocele fluid was used for the control broth tubes.

‡ The drug broth contained Torrey "B" broth enriched with 20 per cent hydrocele fluid and 1-133 final concentration of sulfanilamide.

0 indicates no growth; ++ indicates from 201 to 500 colonies per plate; ++++ indicates over 1,000 gonococcus colonies per plate.

of gonococcal glyceric dehydrogenase activity. It may also be seen that gonococcal glyceric dehydrogenase was inhibited by smaller concentrations of the drugs than was lactic dehydrogenase. Sulfanilamide is a possible exception, since with this compound inhibition of lactic dehydrogenase was slightly greater.

Inhibition of gonococcal catalase. As is shown in table 5, potassium permanganate was found to be the most powerful inhibitor of gonococcal catalase among the compounds tested. Silver nitrate and protargol were also very potent inhibitors, but the other compounds affected the enzyme little or not at all.

Inhibition of gonococcal peroxidase. Silver nitrate, potassium permanganate, and protargol in high dilutions, as shown in table 6, completely inhibited gono-

TABLE 3
*Effect of germicides on gonococcal lactic dehydrogenase**

DRUG	MOLAR CONCENTRATION	PER CENT INHIBITION
Silver nitrate	9.77×10^{-5}	100
Protargol	1.89×10^{-4} (Ag)	100
Neo-silvol	8.17×10^{-3} (Ag)	100
Silver nucleinate	1.79×10^{-1} (Ag)	94
Argyrol	1.85×10^{-1} (Ag)	92
Potassium permanganate	6.32×10^{-4}	100
Merthiolate	2.46×10^{-1}	85
Sulfanilamide	4.36×10^{-2}	26

* Each Thunberg-type tube contained 0.5 ml. of 0.0005 M methylene blue chloride, 0.5 ml. of 0.555 M lactic acid neutralized to pH 7.4 with NaOH, 0.1 ml. of peptone-NaCl solution, 1.9 ml. of M/20 phosphate buffer (Clark and Lubs) pH 7.4, and 1.0 ml. of drug-treated suspension of organisms. The test was conducted at 37°C. Observations were made for a period of 120 minutes.

TABLE 4
*Effect of germicides on gonococcal glyceric dehydrogenase**

DRUG	MOLAR CONCENTRATION	PER CENT INHIBITION
Silver nitrate	1.17×10^{-5}	100
Protargol	1.49×10^{-5} (Ag)	100
Neo-silvol	4.09×10^{-3} (Ag)	100
Silver nucleinate	2.55×10^{-4} (Ag)	100
Argyrol	4.65×10^{-3} (Ag)	100
Potassium permanganate	7.90×10^{-5}	100
Merthiolate	2.46×10^{-1}	100
Sulfanilamide	4.36×10^{-2}	2

* The conditions for this series of experiments were the same as those given in the footnote to table 3, with the exception that 0.471 M glyceric acid neutralized to pH 7.4 was used as the substrate instead of lactic acid.

coccal peroxidase whereas with the other germicides, much higher concentrations were necessary. Sulfanilamide, in the concentration used, had no appreciable effect on peroxidase.

Observations on the relation of gonococcal catalase to peroxidase. It should be noted that the substrate used to test for peroxidase activity consisted of pyrogallol as well as hydrogen peroxide. Since the gonococcus has a strong catalase

activity, the possibility arose that the presence of catalase may interfere with the peroxidase test. Experiments were therefore carried out to determine the effect of gonococcal catalase on the quantitative peroxidase test. A large num-

TABLE 5
*Effect of germicides on gonococcal catalase**

DRUG	MOLAR CONCENTRATION	PER CENT INHIBITION
Silver nitrate	2.94×10^{-3}	100
Protargol	3.78×10^{-3} (Ag)	100
Neo-silvol.	8.17×10^{-3} (Ag)	3
Silver nucleinate ..	1.79×10^{-1} (Ag)	0
Argyrol ..	1.85×10^{-1} (Ag)	8
Potassium permanganate.....	6.32×10^{-4}	100
Merthiolate.....	2.46×10^{-1}	5
Sulfanilamide.....	4.36×10^{-3}	0

* The catalase test was performed in 125 ml. Erlenmeyer flasks containing 26.0 ml. of M/150 phosphate buffer (pH 6.5), 3.0 ml. of N/1 H_2O_2 (diluted from superoxol) and 1.0 ml. of drug-treated acetate suspension of organisms. The test mixture was placed in an ice bath (1° - 2° C.) for one hour, after which 2.0 ml. of 25 per cent H_2SO_4 was added to arrest enzyme activity. The entire contents of the flask was then titrated with standard N/10 $KMnO_4$.

TABLE 6
*Effect of germicides on gonococcal peroxidase**

DRUG	MOLAR CONCENTRATION	PER CENT INHIBITION
Silver nitrate.....	1.96×10^{-3}	100
Protargol	3.78×10^{-3} (Ag)	100
Neo-silvol.	8.17×10^{-3} (Ag)	72
Silver nucleinate	1.79×10^{-1} (Ag)	49
Argyrol ..	1.85×10^{-1} (Ag)	46
Potassium permanganate	6.32×10^{-4}	100
Merthiolate. .	2.46×10^{-1}	78
Sulfanilamide....	4.36×10^{-3}	2

* The peroxidase test was performed in 125 ml. Erlenmeyer flasks containing 21.0 ml. of M/150 phosphate buffer (pH 7.2), 3.0 ml. of N/1 H_2O_2 (diluted from superoxol), 5.0 ml. of 1.56 per cent pyrogallol solution in pH 7.2 M/150 phosphate buffer, and 1.0 ml. of drug-treated organisms. The enzyme reaction was carried out in a 20° C. constant temperature water bath for exactly 15 minutes. Two ml. of a 25 per cent solution of H_2SO_4 were added, the purpurogallin formed was extracted with purified ethyl ether after standing at room temperature for five minutes. The concentration of the dyestuff was determined in a Duboscq type colorimeter compared with a purpurogallin standard prepared by the method of Graebe (1914).

ber of catalase inhibitors mentioned in the literature were tested. The volumetric methods for determining catalase activity advocated by Bailey (1917) and by Morgulis (1921) were employed, since it was found that inhibitors such as

hydroxylamine and formalin interfered with the permanganate titration method. No inhibitor was found which completely inhibited gonococcal catalase without also affecting gonococcal peroxidase activity to a large extent. However, hydroxylamine hydrochloride in a concentration of 9.97×10^{-4} M was selected for the purpose since catalase was found to be completely inhibited whereas peroxidase was inhibited by about 60 per cent (table 7).

Standard suspensions were first subjected to the action of silver nitrate and potassium permanganate in the usual manner so that graded concentrations of the drugs effected the inhibition of peroxidase activity at the endpoint zones. The respective drug-treated suspensions were then tested for peroxidase (pyrogallol) activity with the addition of 9.97×10^{-4} M $\text{H}_2\text{NOH} \cdot \text{HCl}$ to the test mixture. In parallel experiments, comparisons were also made with similar drug-treated suspensions without the addition of hydroxylamine to the reaction mixture. It was found that the same endpoints were obtained with both drugs whether hydroxylamine was added to the test mixture or not.

TABLE 7

*The effect of various concentrations of hydroxylamine hydrochloride on gonococcal catalase and peroxidase**

FINAL CONCENTRATION OF $\text{H}_2\text{NOH} \cdot \text{HCl}$ (M)	INHIBITION OF CATALASE	INHIBITION OF PEROXIDASE
	<i>per cent</i>	<i>per cent</i>
9.97×10^{-5}	32	2
9.97×10^{-4}	100	56
9.97×10^{-3}	100	100

* The conditions for the catalase test are given in the footnote to table 5, with the modification that the volumetric method was used. The conditions for the peroxidase test are given in the footnote to table 6. The H_2NOH solutions were adjusted to pH 7.2. Both catalase and peroxidase tests were conducted at pH 7.2 at a temperature of 20°C. for exactly 15 minutes.

It seemed possible that part of the purpurogallin formed might not have been produced peroxidatively, but instead by the independent oxidative action of gonococci on pyrogallol by some system which did not require hydrogen peroxide. Experiments were carried out to determine how much purpurogallin was formed when pyrogallol was used without the presence of hydrogen peroxide in the enzyme test. The results indicated that under these conditions a negligible quantity of purpurogallin was produced by the action of gonococci on pyrogallol.

Callow (1926) stated that, except for streptococci, the peroxidase system of all bacteria was heat-stable when benzidine or guaiac with hydrogen peroxide were used as substrates. *N. gonorrhoeae* was not included in her studies. In the present study, the heated enzyme controls in the pyrogallol peroxidase tests showed the enzyme to be heat-labile. Following the general methods employed by Callow, it was found that a weak blue color was obtained when both aqueous and acetate suspensions of gonococci were tested with the benzidine-hydrogen peroxide reagent. In both instances, the colors faded within a short period of

time. However, when the suspensions were heated at boiling temperature for a period of 15 minutes, the qualitative benzidine peroxidase test gave a stronger and more lasting color reaction. As rated above, the qualitative pyrogallol peroxidase test was found to be negative when suspensions were heated in the same way. While the addition of KCN to the qualitative pyrogallol peroxidase test using unheated suspensions of gonococci was negative, inconclusive results were obtained when varying concentrations of KCN were added to the gonococcal benzidine peroxidase test mixture. Further work is necessary to establish the true nature of gonococcal peroxidase.

Inhibition of gonococcal indophenol oxidase. The data presented in table 8 show that silver nitrate and protargol are potent inhibitors of indophenol oxidase. Much higher concentrations of merthiolate were required to effect complete

TABLE 8
*Effect of germicides on gonococcal indophenol oxidase**

DRUG	MOLAR CONCENTRATION	PER CENT INHIBITION
Silver nitrate	2.94×10^{-3}	100
Protargol	7.76×10^{-3} (Ag)	100
Neo-silvol	8.17×10^{-3} (Ag)	79
Silver nucleinate	1.79×10^{-1} (Ag)	51
Argyrol	1.85×10^{-1} (Ag)	50
Potassium permanganate	†	†
Merthiolate	2.46×10^{-1}	100
Sulfanilamide	4.36×10^{-2}	55

* The quantitative indophenol oxidase tests were performed in 25 × 100 mm. pyrex test tubes containing 5.0 ml. of the diamine-naphthol reagent, 5.0 ml. of M/20 phosphate buffer of pH 6.6 and 1.0 ml. of drug-treated acetate suspension of organisms. Tests were carried out in a 37°C. water bath for a period of exactly 15 minutes, after which 2.0 ml. of a 2 per cent solution of unadjusted KCN were added. The dyestuff was extracted with 1:1 ethyl alcohol-chloroform mixture and compared in a Duboscq type colorimeter with alpha-naphthol blue standards.

† Could not be determined.

inhibition; with the other drugs, the highest concentration used caused only partial inhibition. The effect of potassium permanganate on gonococcal indophenol oxidase could not be determined accurately since supplementary experiments showed that enough permanganate was present in the washed cells to oxidize the substrate independent of the enzyme activity of the bacteria.

General consideration of the results of the germicidal and enzyme inhibition tests. In table 9, a compilation of the results of the various tests is presented. With the exception of silver nitrate and potassium permanganate, there was no obvious correlation between the concentration of a drug which was lethal for gonococcus and that causing complete inhibition of the various enzymes tested. However, sulfanilamide is a notable exception. Within the limits of solubility of this drug, no apparent effect on viability could be observed during the test period. In line with this finding, there was relatively little effect on the activity

TABLE 9

*Compilation of the results of the germicidal and enzyme inhibition tests on gonococcus**

COMPOUND	VIABILITY TEST	DEHYDROGENASES		CATALASE	PEROXIDASE	INDOPHENOL OXIDASE
		Lactic	Glyceric			
Silver nitrate						
Per cent inhibition	Lethal	100	100	100	100	100
Concentration of drug†	1-800,000	1-60,000	1-500,000	1-2,000	1-3,000	1-2,000
Molarity‡	7.35×10^{-6}	9.77×10^{-6}	1.17×10^{-5}	2.94×10^{-3}	1.96×10^{-3}	2.94×10^{-3}
Protargol						
Per cent inhibition	Lethal	100	100	100	100	100
Concentration of drug†	1-200,000	1-4,000	1-50,000	1-200	1-200	1-100
Molarity‡	3.78×10^{-6}	1.89×10^{-6}	1.49×10^{-5}	3.78×10^{-3}	3.78×10^{-3}	7.76×10^{-3}
Neo-silvol						
Per cent inhibition	Lethal	100	100	3	72	79
Concentration of drug†	1-50,000	1-100	1-200	1-10	1-10	1-10
Molarity‡	1.63×10^{-5}	8.17×10^{-5}	4.09×10^{-5}	8.17×10^{-3}	8.17×10^{-3}	8.17×10^{-3}
Silver nucleinate						
Per cent inhibition	Lethal	94	100	0	49	51
Concentration of drug†	1-80,000	1-10	1-7,000	1-10	1-10	1-10
Molarity‡	2.22×10^{-5}	1.79×10^{-1}	2.55×10^{-4}	1.79×10^{-1}	1.79×10^{-1}	1.79×10^{-1}
Argyrol						
Per cent inhibition	Lethal	92	100	8	46	50
Concentration of drug†	1-50,000	1-10	1-400	1-10	1-10	1-10
Molarity‡	3.71×10^{-5}	1.85×10^{-1}	4.65×10^{-3}	1.85×10^{-1}	1.85×10^{-1}	1.85×10^{-1}
Potassium permanganate						
Per cent inhibition	Lethal	100	100	100	100	§
Concentration of drug†	1-100,000	1-10,000	1-80,000	1-10,000	1-10,000	§
Molarity‡	6.32×10^{-5}	6.32×10^{-4}	7.90×10^{-5}	6.32×10^{-4}	6.32×10^{-4}	§
Merthiolate						
Per cent inhibition	Lethal	85	100	5	78	100
Concentration of drug†	1-900,000	1-10	1-10	1-10	1-10	1-10
Molarity‡	2.73×10^{-6}	2.46×10^{-1}	2.46×10^{-1}	2.46×10^{-1}	2.46×10^{-1}	2.46×10^{-1}
Sulfanilamide						
Per cent inhibition	Not lethal	26	2	0	2	55
Concentration of drug†	1-133	1-133	1-133	1-133	1-133	1-133
Molarity‡	4.36×10^{-3}	4.36×10^{-3}	4.36×10^{-3}	4.36×10^{-3}	4.36×10^{-3}	4.36×10^{-3}

* In the case of neo-silvol, merthiolate, silver nucleinate and argyrol, the drugs could not be used in a concentration higher than 1-10 for reasons of solubility. Similarly, concentrations of sulfanilamide above 1-133 ($4.36 \times 10^{-3}M$) could not be prepared.

† Expressed in terms of dilution of the drugs

‡ Expressed in terms of moles of germicide causing death or inhibition as indicated with the exception of the silver-protein compounds. In the case of the silver-protein compounds the molarity is calculated on the basis of the silver content.

§ Could not be determined accurately.

TABLE 10

The activity of the enzymes at the lethal concentration level of the drugs

DRUGS	LETHAL CONCENTRATION (M)	PER CENT INHIBITION AT LETHAL CONCENTRATION				
		Dehydrogenases		Catalase	Peroxidase	Indo-phenol Oxidase
		Lactic	Glyceric			
Silver nitrate	7.35×10^{-6}	12	62	0	1	4
Protargol	3.78×10^{-6} (Ag)	23	43	0	0	0
Neo-silvol	1.63×10^{-5} (Ag)	34	12	0	0	0
Silver nucleinate	2.22×10^{-5} (Ag)	0	20	0	0	0
Argyrol	3.71×10^{-5} (Ag)	43	36	0	0	0
Potassium permanganate	6.32×10^{-5}	65	63	29	40	*
Merthiolate	2.73×10^{-6}	25	0	0	0	0

* Could not be calculated accurately.

of the enzymes, although it should be pointed out that partial inhibition of indophenol oxidase (55 per cent) and of lactic dehydrogenase (26 per cent) occurred in the presence of the highest concentration of sulfanilamide tested.

Degree of inhibition of enzymes at the lethal concentration of drugs. Thus far, the relationship between the minimal lethal concentration and the smallest amount of drug causing total inhibition of enzyme activity has been considered. It is of value to know what the activity of each enzyme was at the minimal lethal concentration of the drugs. For this purpose, the results of the effect of progressive tenfold dilutions of each drug on the enzymes were plotted. The activity of the enzymes was then calculated graphically at the lethal concentration level of each drug. Table 10 presents the results of these calculations.

It is noted that at the lethal concentration level of the drugs, the dehydrogenases were generally inhibited to a greater extent than were the other enzymes tested. Potassium permanganate was found to produce the most marked effect on the enzymes (indophenol oxidase activity was not calculated because it could not be determined accurately). In contrast to the total enzyme inhibition studies, lactic dehydrogenase activity was inhibited to a greater degree by the action of some of the drugs tested at the lethal concentration level than glyceric dehydrogenase activity at that concentration. The enzymes inhibited most markedly at the lethal concentration of the drugs were lactic dehydrogenase by potassium permanganate, and glyceric dehydrogenase by silver nitrate, as well as by potassium permanganate.

GENERAL DISCUSSION

Certain limitations in an investigation of this nature should be recognized. For example, there was found to be a lack of coincidence between the lethal and the enzyme inhibition endpoints (table 9). This is particularly apparent from a study of table 10, where the degree of inhibition of the enzymes at the lethal concentration of the chemical agents is shown. Several contributing factors may be considered to explain why total inhibition of the enzymes studied does not take place at the lethal concentration of the drugs.

There exists the possibility that the various germicides tested cause the death of gonococcus by inhibiting an enzyme system for which no test was made in the present study. Other fundamental processes, such as the phosphorylating and glycolytic systems, may be limiting factors whose inhibition affects more directly the viability of gonococcus.

Manometric studies have shown a close relationship to exist between the effect of chemical agents on the respiration and on the viability of bacteria (Ely, 1939; Greig and Hoogerheide, 1941). In fact, Bronfenbrenner, Hershey and Doubly (1939) have concluded that bacteriostatic dilutions of germicides depress the oxygen uptake of bacteria by only about 10 per cent, whereas effective germicidal concentrations do so in excess of 80 per cent. It should be noted that the manometric results were expressed as depression of oxygen uptake of the organisms by the action of the chemical agents tested. This really represents the sum total effect of the drugs on the function of several enzymes

in a system necessary to oxidize a particular substrate. In this instance, glucose and horse serum were used in the reaction mixture.

Various mechanisms, whether accessory to the function of the respiratory enzymes or not, may operate and complicate the correlation of enzyme activity with the death of the cell. Fildes (1940), for instance, has come to the conclusion that mercury combines with the sulfhydryl compounds present in *E. coli*; this effect is reversed by the addition of —SH compounds to the suspension. Casman and Rettger (1933) have shown that catalase and succinic dehydrogenase in certain organisms tested may actually inhibit peroxidase activity (qualitative benzidine test).

The present conception of cellular respiration is essentially a transfer of electrons from foodstuffs (substrates) to molecular oxygen by various respiratory enzymes (Barron, 1939). The dehydrogenases occupy a primary position in the respiratory chain of electron transport while cytochrome (indophenol) oxidase plays the terminal role in the series of respiratory enzymes and intermediate carriers. Despite the accumulation of knowledge in regard to the biochemistry of catalase and peroxidase, the real physiological function of these two enzymes is still considered obscure (Lipton, Arnold and Berger, 1939; Green, 1940). These heme catalysts are sometimes regarded as "secondary respiratory enzymes" because of their incidental function in the respiratory chain (Werkman, 1939).

When the effect of drugs on the enzymes is considered, the present investigation shows that the dehydrogenases are the most labile enzymes, while oxidase, catalase and peroxidase are found to be comparatively more resistant to the action of these compounds. It may also be recalled that Casman and Rettger (1933) found succinic dehydrogenase to be distinctly inhibited at the maximum temperature of growth, whereas catalase and oxidase varied considerably in this respect. The work of Haas, Harrer and Hogness (1942) is of interest in this connection. These workers tested the effect of 1×10^{-3} M 2,4-dinitro-o-cyclohexylphenol upon isolated components of the respiratory system of yeast. This chemical agent inhibited *Zwischenferment* (hexosemonophosphoric dehydrogenase) and the total respiration of yeast almost completely, while cytochrome *c* reductase (an intermediary flavoprotein functioning between hexosemonophosphate and cytochrome *c*) was inhibited by 70 per cent. Cytochrome oxidase was not affected at all by this compound. In general, the respiratory enzymes functioning at the beginning of the electron transport chain appear to be affected most readily by the action of the chemical compounds tested.

In the present investigation, the gonococcus was shown to contain both heat-labile pyrogallol and heat-stable benzidine peroxidase activity. The general property of thermostability of bacterial peroxidases was pointed out by Stapp (1924) and Callow (1926). They used the guaiac and benzidine tests. In addition, Stapp found bacterial peroxidases to be resistant to the action of acid, alkali, salts and narcotics. This led Oppenheimer (1926) to conclude that Stapp was working with a purely chemical oxidation system and not with an enzyme. It has been pointed out by Green (1940) that practically all Fe porphyrins can

act as heat-stable peroxidases. Stephenson (1939) has suggested that many peroxidases reported in bacteria are probably of this type.

SUMMARY AND CONCLUSION

The effect of eight germicides on *Neisseria gonorrhoeae* has been studied in an attempt to compare the loss of viability with the inhibition of five respiratory enzymes. The drugs tested were silver nitrate, protargol, neo-silvol, silver nucleinate, argyrol, merthiolate, potassium permanganate and sulfanilamide. The enzymes selected for the inhibition studies were lactic and glyceric dehydrogenases, catalase, peroxidase and indophenol oxidase. Within the limits of the experiments, the following conclusions seem warranted:

1. Death of the cells occurred before significant enzyme inhibition took place. A possible exception to this observation was found in the case of silver nitrate and potassium permanganate. With both of these compounds, the minimal concentration causing complete inhibition of glyceric dehydrogenase corresponded very closely to the lethal concentration levels of these drugs. However, at the lethal concentration of these two chemical agents, approximately 65 per cent inhibition of glyceric dehydrogenase activity was found.

2. Of the eight germicides studied, merthiolate, silver nitrate, protargol and potassium permanganate showed the highest lethal activity on gonococcal suspensions.

3. On the basis of total inhibition, silver nitrate, potassium permanganate and protargol were the most effective enzyme inhibitors. At the lethal concentration of the compounds, potassium permanganate and silver nitrate were found to be the most active enzyme inhibitors. Glyceric and lactic dehydrogenase activities were generally inhibited by chemical agents in smaller concentrations than those of the other enzymes tested.

4. The effect of sulfanilamide on gonococci places this drug in a different category from the other compounds tested. No apparent effect on viability was observed within the 20-minute test period, although exposure of the drug to the organisms for a period of 24 hours caused the death of the cells. Furthermore, the inhibition of respiratory enzymes by sulfanilamide was *nil* with the exception of indophenol oxidase and lactic dehydrogenase. These enzymes were inhibited by 55 per cent and 26 per cent respectively at the highest concentration of the drug tested.

5. The presence of gonococcal catalase has been shown not to interfere with the results obtained in tests for peroxidase activity of gonococci.

6. When pyrogallol with hydrogen peroxide was used as the substrate, a heat-labile peroxidase could be demonstrated with gonococcus. However, the use of benzidine and hydrogen peroxide revealed heat-stable peroxidase-like activity.

7. A quantitative, colorimetric, indophenol oxidase test has been developed for use in studies with the gonococcus.

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A STUDY OF LUMINOUS BACTERIAL CELLS AND CYTOLYSATES WITH THE ELECTRON MICROSCOPE

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INTRODUCTION

The cytolytic phenomena of marine luminous bacteria make them of more than usual interest for study with the electron microscope. Quantitative data on osmotic properties and physiological activity, together with microscopic observations of morphology, have led to the view that the normal cells have a high internal osmotic pressure, amounting to some 22 atmospheres, and that they are surrounded by a strong, rigid wall. At a critical low tonicity of the external medium, this wall is thought to rupture or crack, releasing some of the cell contents. There is an immediate loss of luminescence, respiration, motility, and viability (Harvey, 1915; Hill, 1929; Korr, 1935; Johnson and Harvey, 1938). The suspension partially clears, and surface-active constituents showing a specific precipitin reaction are liberated (Johnson, 1941). The volume of cells which can be precipitated in the ultracentrifuge becomes less, but direct microscopic counts of "ghosts" visible under the dark field indicate that, within the accuracy of counting, none of the cells completely dissolve (Johnson and Harvey, 1937).

Electron micrographs should provide more detailed evidence than has hitherto been available concerning the structural changes accompanying this "osmolysis" of the cells, and its possible relation to lytic phenomena brought about by other agents. Furthermore, the ready ease of obtaining "ghosts," presumably consisting of a firm outer shell, but more or less devoid of cell contents, offers certain advantages for the investigation of component parts of normal cells. On the other hand, an intrinsic disadvantage is encountered in the fact that the cells are "normal" only in a medium of relatively high salt content, and they immediately cytolyze when suspended in distilled water instead of isotonic 3 per cent NaCl solution. The presence of considerable salt greatly interferes with obtaining satisfactory electron micrographs. To some extent, the difficulty has been surmounted by special methods, and by studying a variety of luminous species, including non-halophilic types, embodying a wide range of susceptibility to osmotic cytolysis.

MATERIALS AND METHODS

The following species have been studied: *Achromobacter fischeri*, *A. harveyi* (Johnson and Shunk, 1936), *Photobacterium phosphoreum*,¹ *P. pierantonii*,¹ *P.*

¹ Cultures of these organisms were obtained in 1939 from the Delft Collection, through the kindness of Professor A. J. Kluyver.

splendidum,¹ *P. sepiæ*,¹ *Vibrio albensis*,¹ and *V. phosphorescens*.² The first six of these are marine species and were cultivated on 3 per cent NaCl nutrient agar containing 1 per cent glycerol and 0.5 per cent CaCO₃. *P. phosphoreum*, a psychrophilic type, was cultivated at 15°C., but the others were incubated at room temperature, varying between 20 and 25°C. The last two species in the list are non-halophilic, although the luminescence of *V. phosphorescens* rapidly disappears in distilled water. *V. albensis*, closely related to it, is apparently more hardy. Both were cultivated at room temperature on the same medium as the marine species but containing only 0.9 per cent rather than 3 per cent NaCl.

For the electron micrographs, the suspension was prepared by introducing a loopful of the growth from a brightly luminous agar slant into 1 ml. of distilled water, salt solution, or other medium described below. A drop of this suspension was then added to the collodion membrane in the usual manner and dried quickly.

RESULTS

"Normal" cells

With halophilic species, e.g., *A. fischeri*, which is especially sensitive to distilled water cytolysis, satisfactory micrographs were obtained by allowing a thin droplet of a suspension of the cells in 3 per cent NaCl to dry on the membrane. Heavy salt crystals were always abundant, but in favorable spots, individual cells could be found separate from, and unobscured by the crystals (figs. 1, 2, 3, 4, 6). It would appear likely that some shrinkage of the cell occurs as the film dries, and the concentration of salt increases. Volume studies with the ultra-centrifuge (Johnson and Harvey, 1937) indicate that the total shrinkage would not amount to more than 30 per cent of the normal cell volume. The electron micrographs indicate that any such shrinkage must be quite uniform and not in the nature of a plasmolysis. Distinct cell walls, such as those described by Mudd *et al.* (1941) are not apparent in these preparations. The osmotic properties referred to above, however, indicate that strong walls normally surround the cells. The ultimate constitution of the wall, and its relation to the protoplast under various conditions, remain to be elucidated. In the present instance it is possible that such a structure, even though present, might be so closely applied to the dehydrated protoplasm, and either so thin or so nearly of the same density, that it is indistinguishable as a separate component of the cell body. Furthermore, structures apparent in distilled-water cytolysed (e.g., figs. 44, 45, 49) and in specially treated specimens (figs. 12, 17, 18, 23, 27, 29) more or less clearly indicate the presence of cell walls.

The cells of halophilic species less sensitive to osmolysis, e.g., *P. phosphoreum*, appear similar, apart from minor differences in density, whether dried from a 3 per cent NaCl or a distilled water suspension (figs. 7, 8, and 9), even though the luminescence is immediately extinguished in the latter. In 0.73 molar sucrose, which maintains the osmotic pressure of the medium without NaCl

¹ Original culture was kindly supplied by Professor M. H. Soule.

or other electrolytes, luminescence persists, and the cells may be "fixed" by AgNO_3 , then washed with distilled water, after the method of Mudd and Anderson (1942). Some of the preparations treated in this manner revealed the presence of peculiar, vesicular out-pocketings of the periphery of the cell (figs. 13 and 15). Their exact nature is not clear, although it is possible that they are capsular structures, inasmuch as the Burri method of staining indicates that capsules appear to be formed by this species. No structure quite so prominent was found following similar treatment of other species, although membranous extensions and cell wall-like structures were frequently evident (figs. 12, 17, 18).

In effort to obtain pictures of normal cells, fixation by other agents was tried, including alcohol of various concentrations. When the cells of *A. fischeri* were suspended directly in 95 per cent alcohol, a rapid crystallization, probably of NaCl , took place, leaving numerous minute crystals adjacent to each cell. In addition, a peripheral structure of the cell apparently comes off and dries as a thin, wrinkled, membrane-like object, relatively transparent to the electron beam (fig. 10). The appearance of this structure suggests that it may be identical with that observed in some of the distilled water cytolysates (figs. 42, 46, and 52). Furthermore, from the evidence available, it must represent either the cell wall itself, according to the current understanding of the term, or some component part of it. The protoplast remains dense. It is fairly dense also in 60 per cent alcohol, which evidently allows the crystals to form more slowly and become larger (fig. 11). Crystals were sometimes observed similarly disposed in preparations made in distilled water (fig. 24).

In all cases described above, the protoplast appears dense and reveals little evidence of internal structure. These results are in accord with previous observations concerning the "normal" cells of a number of bacterial species (Piekarski and Ruska, 1939; Jakob and Mahl, 1940; Mudd and collaborators, 1942).

The non-halophilic species of luminous bacteria, viz. *V. albensis* and *V. phosphorescens* do not undergo cytolysis so readily as the marine organisms. Distilled water preparations nevertheless indicate certain structural features similar to those which are apparently induced by distilled water treatment of marine species, described below. These features include all gradations of internal appearance from a fairly dense, solid structure, to apparently empty "ghosts." Internal, dense spheroid bodies are frequently observed (fig. 21) recalling structures of similar appearance which have been previously noted by Piekarski and Ruska, and by Mudd. Figures 21, 22, 23, 27, 28, 29, 30 represent typical electron micrographs of both species without special treatment, as well as "stained" and "fixed" preparations, and cells in normal and immune serum. Various degrees of cytolysis are apparent in the shrinkage (fig. 22), coagulation (fig. 23), and partial disintegration (fig. 29) of cells. It is interesting to note that dilute nigrosin is apparently accumulated by the protoplast, but apparently less so by the cell wall, thus leaving the latter distinctly visible (fig. 27). A rather similar appearance, from a different cause, may result when the cells are agglutinated in homologous antiserum (fig. 28). In heterologous antiserum the appearance is different (fig. 30).

Dilute phenol markedly destroys the normal appearance of the cell, apparently dissolving as well as coagulating some of the constituents (fig. 19). Bichloride of mercury evidently precipitates evenly (fig. 20). Internal structure, apparent in the distilled water preparations, is partially obscured by the HgCl_2 precipitate.

Distilled water cytolysis of marine species

Plates IV, V and VI include typical electron micrographs obtained of distilled water suspensions of *A. fischeri* and of *A. harveyi*. Both species are sensitive to osmotic pressure changes, and the former, in particular, has been extensively investigated with respect to the relation of specific salts and osmotic pressure to physiological activity. An exudation of cell contents apparently takes place when the cells are suddenly immersed in distilled water, leaving ghosts in various stages of disintegration. Figures 31, 32 and 33 possibly represent an early stage in the exudation of contents, although the actual lytic process is no doubt very rapid in any given cell, judging from the rapidity of changes in physiological activity. Such an escape of cell contents recalls the observations made by Wamoscher (1930) in his micro-dissection experiments. In general, allowing the cells to remain in distilled water for half an hour before examining appears to make very little difference, in comparison to the picture obtained when the specimen is dried as quickly as possible (2 or 3 minutes) after preparation. The wall sometimes "peels off" (figs. 34, 42, and 52) in the manner observed after suspending the cells in 95 per cent alcohol (fig. 10). Flagella sometimes remain attached in spite of this loss of outer structure (figs. 42 and 52). Finally, a more or less completely empty "ghost," quite transparent to the electron beam, may remain. The flagella may likewise remain attached to this outer remnant (figs. 36, 37, 39, 43). Spheroid bodies of considerable density, are sometimes seen at one or both poles of an otherwise practically empty ghost (fig. 38).

The cytolysis of *A. harveyi* (Plate V) appears to be essentially the same as that of *A. fischeri*. The ghosts in some cases indicate that the polar region has been disrupted (fig. 41) and in nearly all cases show a mosaic density over the whole surface or body. The "transparent" areas, where visible as individual units in this mosaic, tend to be roughly circular, varying to elliptical in shape. The diameters extend from as little as 5.6 millimicrons to 84 $\text{m}\mu$ and larger. The latter probably represent a confluence of smaller units. The former are well within the range of diameters of protein molecules (Stanley and Anderson, 1942). The end-product of cytolysis again appears to be a cell practically empty of contents (fig. 43), although the flagella may persist. Treatment of such preparations with AgNO_3 indicates that much of the material precipitated by this reagent is now on the outside rather than inside the cells (fig. 14).

Flagella and other structures

The figures in Plates III, IV and V, show certain details that are interesting in connection with the structure of flagella. Figures 36, 37, 39, and 43 show flagella attached to cell walls more or less devoid of normal contents. Figures

42 and 52 indicate flagella attached to the cell, after the wall has partly come off. It would thus appear that elements continuous with both the wall and protoplast form parts of the flagella, a circumstance which would lend support to both



FIG. 52. PARTIAL GHOST OF *A. FISCHERI* IN DISTILLED WATER

It appears that the cell wall has peeled off of approximately one half the cell, leaving the naked protoplast, with flagella attached at one end. At the opposite end, the cell wall remains in place. The thin, crumpled structure at one side appears to be continuous with the cell wall, and for this reason, as well as its evidently similar density, possibly represents a portion of the wall that has come off. Note that the diameter of the flagella varies, according to recognizable differences in the number of longitudinal components in certain regions. See also figures 36, 37, 39, 40, and 42, as well as discussion in the text.

sides of the early controversy regarding the origin of these structures (reviewed by Reichert, 1909; see also Knaysi, 1938; Lewis, 1941). The precise relations, however, cannot be designated further, since the exact interpretation of the elec-

tron micrographs remains subject to some unavoidable uncertainty without collateral evidence.

Figure 40 is of particular interest in regard to flagella. There appear to be two distinct types: larger ones, with a diameter of approximately 0.04 micron, and more numerous smaller ones averaging 0.016 micron in diameter. To all appearances, the former, as well as the latter, are individual flagella. Figures 36, 37, 39, 42 and 52, however, indicate clearly that a number of flagella sometimes stick together in such a manner that, in spite of the resolving power of the electron microscope, they appear to be fused into a single longitudinal structure throughout a whole or part of their lengths. For this reason, there is some question regarding the thickness of the smallest unit. The varying diameters of longitudinal components, together with the occasionally observed, fairly regularly dispersed nodes of greater density down the lengths of the flagellum (fig. 43) bear certain resemblances to the structure of collagen (Schmitt *et al.*, 1942). The points in common, however, may be entirely superficial.

Internal structures worthy of note are apparent in Plate VI, although their significance remains to be elucidated. Figure 51 is especially striking because of its coiled thread of dense material. Though less regular than the one in *Thiobacillus thiooxidans* shown by Umbreit and Anderson (1942), possibly it is a related structure. On the basis of appearance, and of analogy with higher organisms it is tempting to conjecture that some of these structures represent a phase in cell division.

The ghosts in figures 48 and 50 show roughly circular, relatively transparent spots resembling those apparent in ghosts of *A. harveyi* and *A. fischeri* already referred to, and in addition some long and narrow slit-like areas. On the basis of appearance alone the significance of these areas is not clear. As pointed out above, however, indirect evidence from several entirely different approaches, viz. immunological reactions; osmotic and surface properties; dark-field microscopy and counts of ghosts; changes in physiological activity, etc; indicates that distilled water cytotoxicity is accompanied by an exudation of protoplasmic constituents through cracks or openings too small to be seen by ordinary methods with the light microscope. Possibly the clear areas, both circular and elongated, represent the site where such an exudation has taken place as a result of the rupture or dislocation of normal constituents during the process of cytotoxicity.

Further study should make it possible to establish more precise correlations between cell structure and specific functions. The foregoing results substantiate the general view, previously advanced on the basis of both quantitative and qualitative evidence, concerning the phenomenon of osmotic cytotoxicity. Variations in susceptibility among different species, and in the detailed picture of a single species, are no more than would be expected. In some respects, the property of osmotic toxicity is an advantage in investigating internal structure. The luminous bacteria should provide favorable material for further study.

It is a pleasure to acknowledge our indebtedness to Mr. Karl Seiler for his valuable assistance in making the electron micrographs.

SUMMARY

Electron micrographs have been obtained of eight species of luminous bacteria, including *Achromobacter fischeri*, *A. harveyi*, *Photobacterium phosphoreum*, *P. pierantonii*, *P. sepiac*, *P. splendidum*, *Vibrio albensis*, and *V. phosphorescens*, the latter two of which are "fresh water" and the others marine.

Cells of the marine species, prepared from an isotonic 3 per cent NaCl suspension are dense, revealing hardly any evidence of internal structure, or distinct cell wall. Suspensions of the cells in distilled water, reveal evidence of cytolysis involving an exudation of cell contents, and partial disintegration of the wall. The ghosts reveal a mosaic density, with relatively transparent, roughly circular, or slit-like areas. The smallest clearly recognizable areas of this sort are approximately 5 millimicrons in diameter.

Flagella frequently remain attached to ghosts, and sometimes to the body of semi-ghosts, whose outer wall has remained on half of the cell. The diameter of the flagella varies with the number of longitudinal units. In *A. harveyi*, two distinct flagella types, averaging 10 and 16 millimicrons, respectively, are encountered on the same cell. The larger ones possibly represent a single structure, although it is also possible they represent several longitudinal units.

Differences were noted in the susceptibility of marine species to distilled water cytolysis. The fresh water species were fairly resistant but gave evidence of changes similar to those of the halophilic types when immersed in distilled water.

Fixation, by AgNO_3 , of halophilic cells in isotonic sucrose revealed capsule-like structures on *P. phosphoreum*. In 95 per cent alcohol, the wall of *A. fischeri* crumpled off. Dilute phenol apparently dissolved as well as coagulated *V. albensis* cells. Dilute nigrosin made it possible to distinguish cell wall and protoplast of *V. albensis*.

Internal structures, such as those previously noted in electron microscope studies, are shown in distilled water suspensions of both marine and fresh water species.

The detailed picture of distilled water osmolysis, revealed by the electron microscope, is in general accord with the views previously advanced on the basis of changes in physiological activity, cell volume, surface properties, and immunological reactions.

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PLATE I

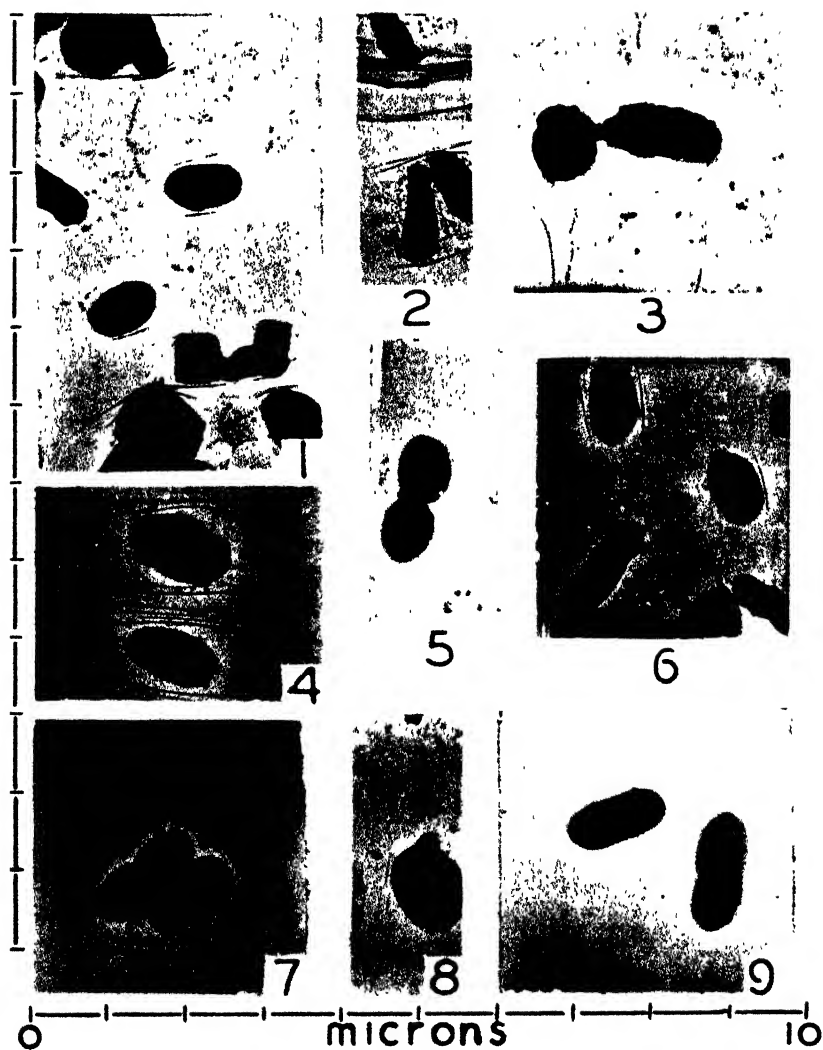
FIG. 1. *A. fischeri* after drying from a suspension in 3 per cent NaCl. Dense cells and salt crystals of about the same size.

FIGS. 2, 3, 4 AND 6. *A. fischeri* in 3 per cent NaCl.

FIG. 5. *P. sepiæ* in distilled water.

FIGS. 7 AND 9. *P. phosphoreum* in distilled water.

FIG. 8. *P. phosphoreum* in 3 per cent NaCl.



(F. H. Johnson, N. Zworykin and G. Warren: Cell study with electron microscope)

PLATE II

FIG. 10. *A. fischeri* after suspending directly in 95 per cent alcohol. The cell wall has partially crumpled away.

FIG. 11. *A. fischeri* in 60 per cent alcohol. The crystals adjacent to the cells are probably NaCl.

FIG. 12. *A. harveyi* suspended in isotonic (0.73 M) sucrose, then treated with approximately 0.25 M AgNO_3 for 30 seconds. Note shrunken protoplasm which has picked up the stain, and the relatively transparent cell wall.

FIGS. 13, 15 AND 16. *P. phosphoreum* in isotonic sucrose, stained as cells of fig. 12, with AgNO_3 . The structure extending out from the dense cell body is present on most of the cells, but occasionally absent.

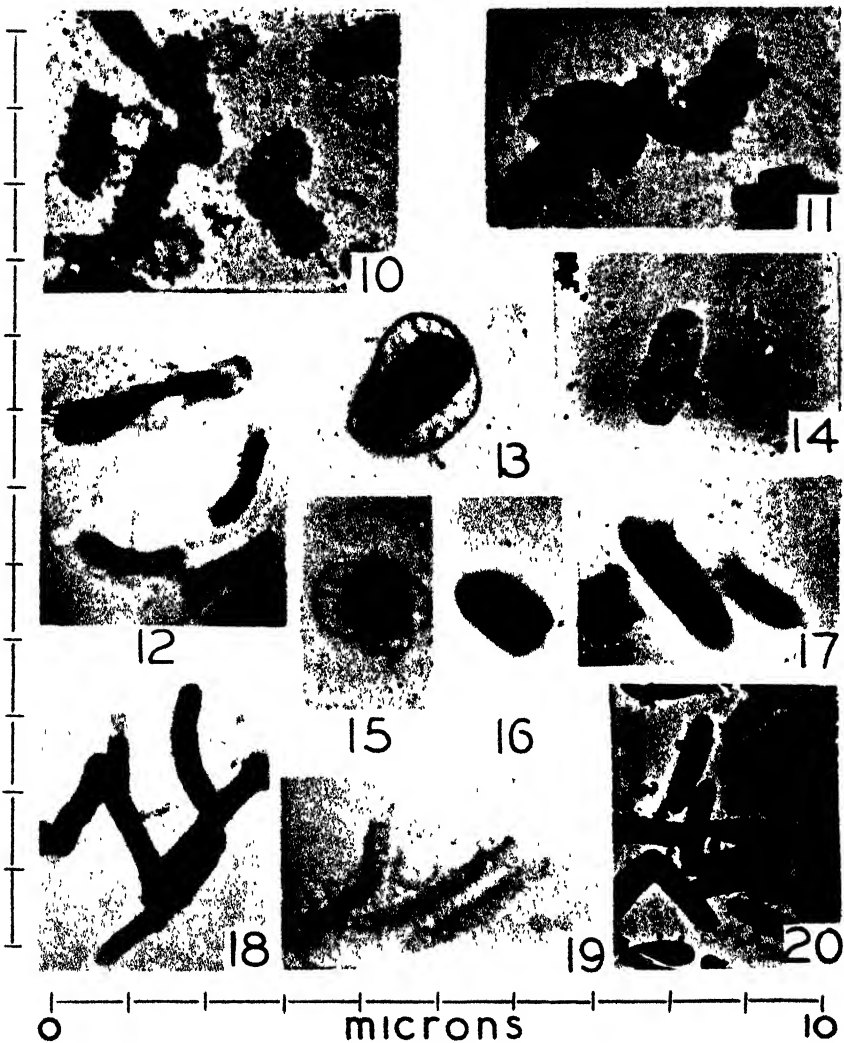
FIG. 14. *A. harveyi* suspended in distilled water, then treated with AgNO_3 as in fig. 12. Relatively slight Ag precipitation on the ghost. The small, dense particles probably include some heavily stained cell contents.

FIG. 17. *A. harveyi*, as in fig. 12, but stained less heavily with AgNO_3 .

FIG. 18. *V. albensis* in distilled water, stained 30 seconds with approximately 0.5 M AgNO_3 . Note protoplasm shrunken away from the wall only at ends of cell.

FIG. 19. *V. albensis* in distilled water, treated briefly with 0.5 per cent phenol.

FIG. 20. *V. albensis* in distilled water, stained for a few seconds with 1:1000 HgCl_2 .



(F. H. Johnson, N. Zworykin and G. Warren. Cell study with electron microscope)

PLATE III

FIG. 21. *V. albensis* in distilled water

FIG. 22. *V. phosphorescens* in distilled water

FIG. 23. *V. phosphorescens* in distilled water. Partially cytolysed cells. Note that flagella are apparently continuous with cell body.

FIG. 24. *P. sepiac* in distilled water. This species resembles *P. phosphoreum* in cytolytic behavior, and in morphology except that the latter is non motile

FIG. 25. *P. pterantonu* in distilled water. In morphology and cytolytic behavior, this species, and also *P. splendidum* (fig. 26), resemble *A. harveyi*

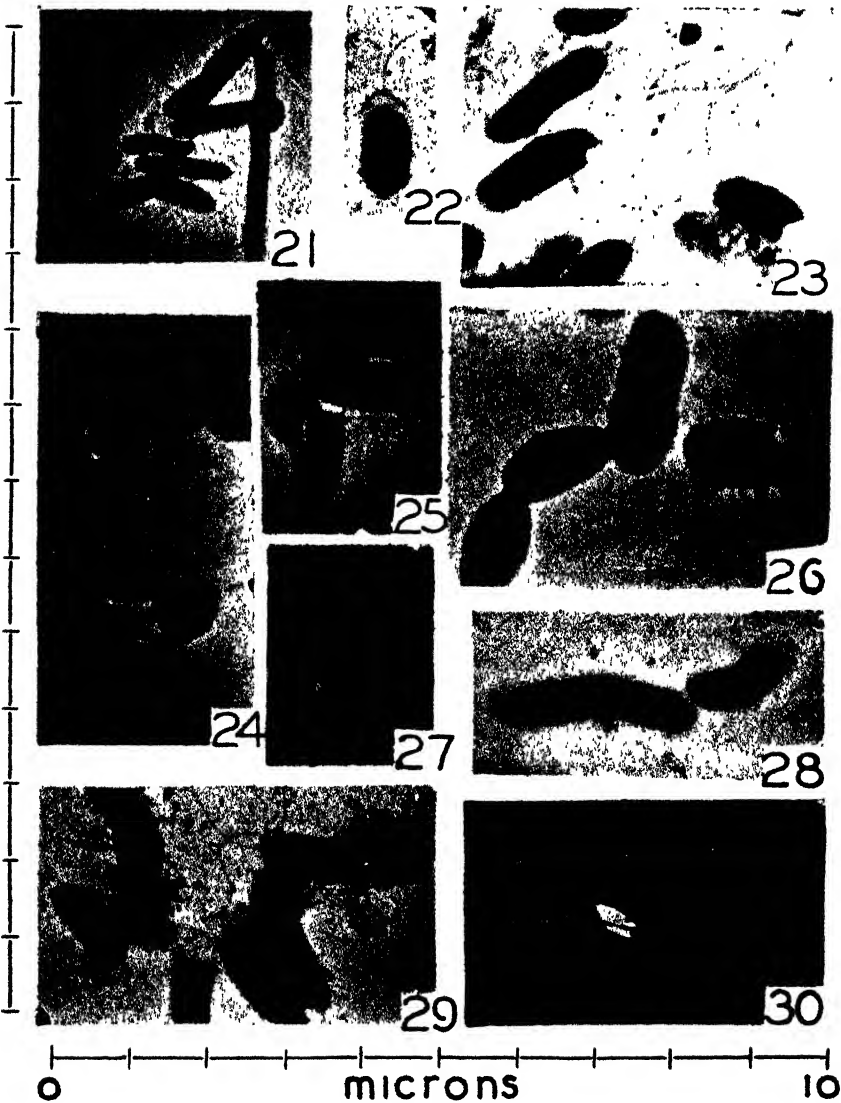
FIG. 26. *P. splendidum* in distilled water

FIG. 27. *V. albensis* in distilled water, stained with dilute nigrosin.

FIG. 28. *V. phosphorescens* agglutinated with homologous antiserum, 1:5, then diluted 1:500 for making the micrograph. The antiserum titer was over 1:10,000

FIG. 29. *V. phosphorescens* in distilled water, showing various degrees of cytolysis

FIG. 30. *V. phosphorescens* in heterologous antiserum. Preparation was made at the same time, using the same suspension of cells, as in fig. 28



(F. H. Johnson, N. Zworykin and G. Warren: Cell study with electron microscope)

PLATE IV

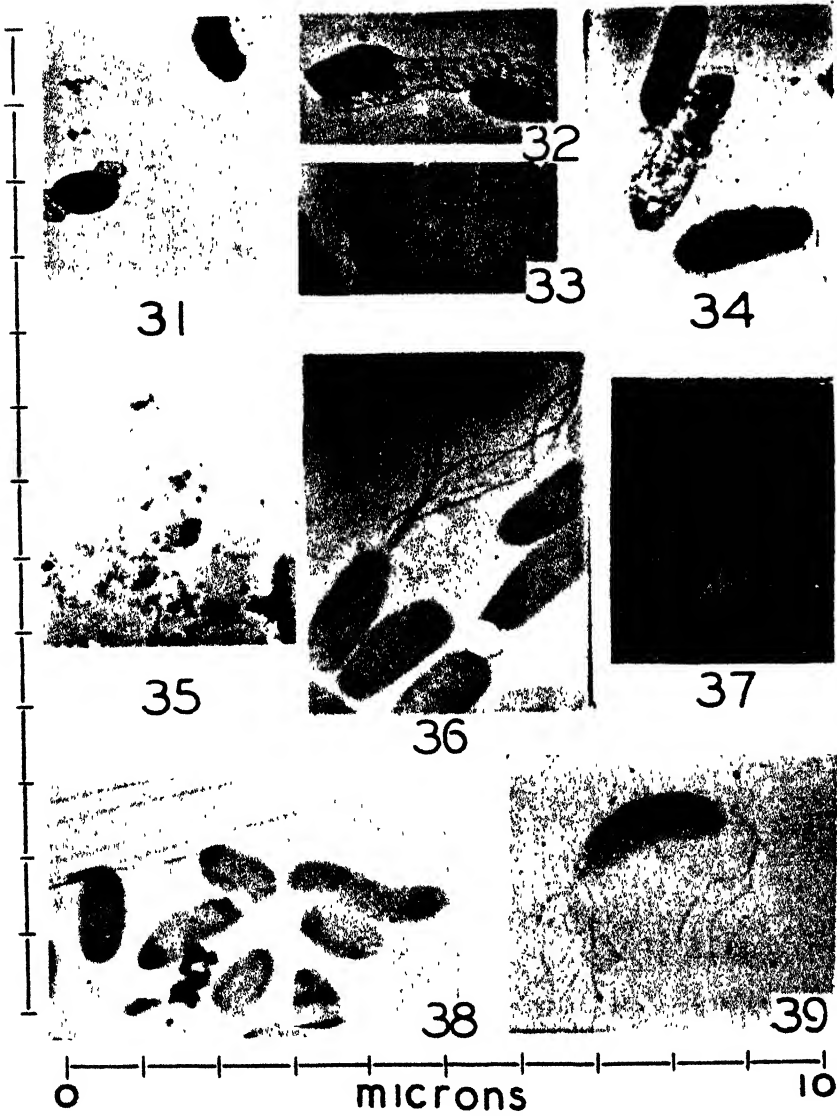
FIGS. 31, 32 AND 33. *A. fischeri* in distilled water, possibly indicating exudation of contents in osmotic cytolysis.

FIG. 34. *A. fischeri* in distilled water. One normal cell, and two other cells in different stages of cytolysis.

FIG. 35. Ghost of *A. harveyi* after twenty minutes in distilled water.

FIGS. 36, 37 and 39. Ghosts of *A. fischeri* cytolysed in distilled water.

FIG. 38. *A. fischeri* cytolysed in distilled water. Note dense bodies in some of the ghosts.



(F. H. Johnson, N. Zworykin and G. Warren: Cell study with electron microscope)

PLATE V

FIG. 40. *A. harveyi* in distilled water. There appear to be two distinct types of flagella, with respect to diameters. This picture was taken with the new, small electron microscope at the R. C. A. Laboratories, Princeton, New Jersey.

FIG. 41. Semi-ghosts of *A. harveyi* in distilled water. Note cell that has apparently burst at one end, and the mosaic density.

FIG. 42. *A. fischeri* in distilled water, showing two incompletely cytolysed cells with outer walls partially off.

FIG. 43. *A. harveyi* in distilled water. Note flagella coming from empty ghost. The significance of the nodes along the flagellae is uncertain (see text). They are clearly not artifacts in the photographic plate, as shown by the uniformity of the picture at the right of the broken membrane.



(F. H. Johnson, N. Zworykin and G. Warren. Cell study with electron microscope)

PLATE VI

FIG. 44. *A. fischeri* in distilled water. Note cell wall and shrunken protoplasm.

FIG. 45. *A. fischeri* in distilled water, showing internal structure in a semi-ghost.

FIG. 46. *A. fischeri* in distilled water.

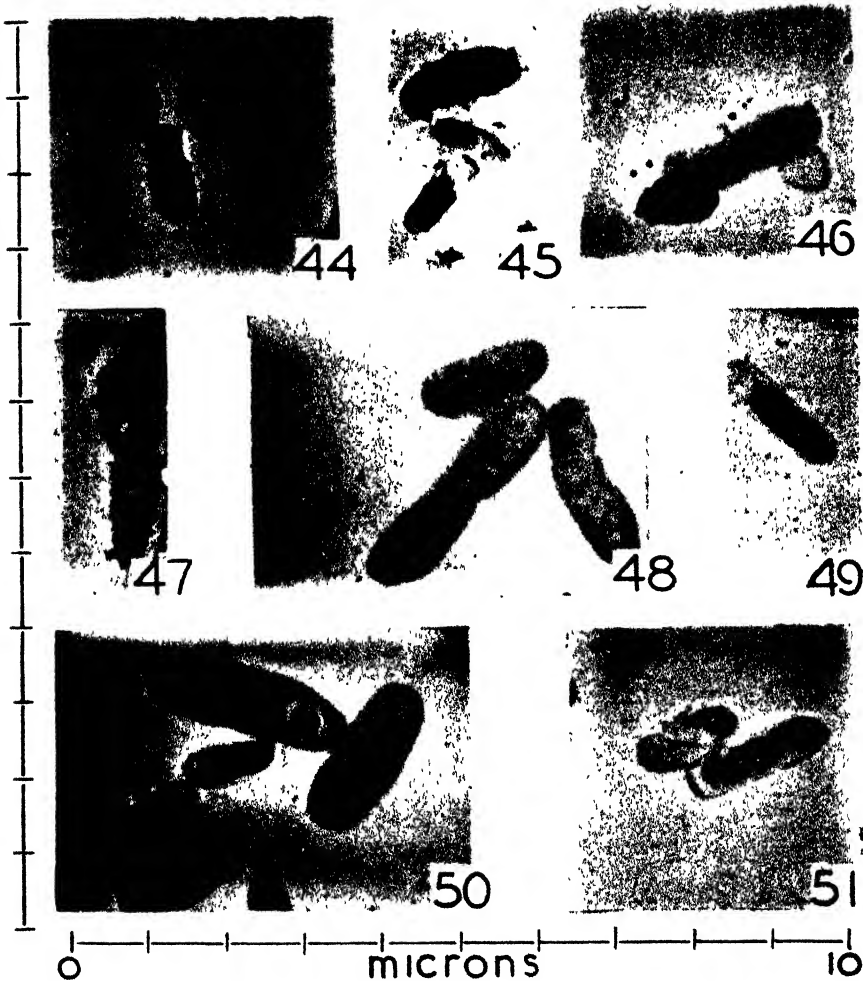
FIG. 47. Partially cytolysed cell of *A. fischeri* in distilled water.

FIG. 48. *A. harveyi* in distilled water. Note mosaic density and apparent "cracks" in the structure.

FIG. 49. *A. harveyi* in distilled water. Semi-cytolysed cell.

FIG. 50. *A. harveyi* in 1 per cent NaCl. Note structure of protoplasm in incompletely cytolysed cell.

FIG. 51. *A. harveyi* in distilled water. A spiral structure of dense material is evident within the partially cytolysed cell.



(F. H. Johnson, N. Zworykin and G. Warren: Cell study with electron microscope)

MICROBIOLOGICAL ASPECTS OF PENICILLIN

I. METHODS OF ASSAY

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Available publications on penicillin (Fleming, 1929; Chain, *et al.*, 1940; Abraham, *et al.*, 1941, 1942; Hobby, *et al.*, 1942; McKee and Rake, 1942; Catch, *et al.*, 1942, Meyer, *et al.*, 1942) indicate that it has excellent prospects as a chemotherapeutic agent and that its microbiological production and chemical purification and isolation are formidable problems. Aside from the report of the Oxford group (Abraham, *et al.*, 1941), little information pertaining to the microbiological aspects of penicillin has appeared. Comprehensive investigations on various phases of the problem have been carried out in this laboratory and considerable experience has accumulated which is worth reporting at this time.

The matter of establishing with some degree of certainty and accuracy the potency of penicillin solutions is one of the most pressing issues confronting workers engaged in penicillin studies. Among various laboratories there are wide divergencies in penicillin assay methods, which, in a good many instances, make it virtually impossible to interpret findings from one laboratory in terms of those from another. The critical studies outlined in this paper are based on experience with all the different types of assays and the conclusions presented are the result of the daily handling for approximately a year of 50 to 100 assays under practical working conditions. Fleming (1942) has already emphasized some features of certain procedures used in penicillin testing, but these are largely qualitative in nature and do not deal with the quantitative aspects considered in the present paper.

METHODS OF ASSAY

Requirements of a penicillin assay

These may vary according to the particular interest and need for accuracy of the individual worker. The main objective is to determine, as accurately as required, the antibacterial activity of a penicillin sample. Depending on the particular objective and the nature of the experiment under way, a high degree of accuracy may not be necessary, and thus many factors conducive to precision are dispensable. On the other hand, detection of relatively small differences in potencies is often required and precautions must be taken to insure this order of sensitivity.

In certain types of biological experiments, such as in measuring blood levels and excretion and pharmacological studies, values accurate to within 100 per cent may be satisfactory. Studies on chemical fractionations, stabilities, purification, and recovery yields from large scale production batches create.

however, an urgent need for the "ideal" assay. A 20 per cent variation may mean quite a difference in the interpretation of a fractionation or extraction process or of a stability experiment.

The results on any given preparation must be reproducible in the same and different laboratories, and, of course, a common language for expressing the activity of penicillin preparations must be used.

Samples should not require pre-treatment to make them suitable for assay as pre-treatment becomes a great handicap where numbers of assays are run routinely.

Any method finally selected as filling the above requirements must be amenable to the running of large numbers of tests in routine.

Results should be available in as short a time as possible after the assay is set up, and the reading should be unequivocal.

The distinctive characteristic of penicillin is its ability to inhibit the normal growth of certain bacteria. At present, the only criterion of potency is the magnitude of the bacterial growth inhibition caused by penicillin preparations. The growth inhibition in certain media is proportional to the concentration of penicillin, and since no competitive phenomenon analogous to para-amino-benzoic acid reversal of sulfonamide action is known, all methods of assay center around determining the smallest amount of penicillin which will cause an arbitrarily established degree of inhibition of the growth of a susceptible test bacterium. In essence, all the proposed methods differ in the mechanics used to measure the inhibition.

Entirely fallacious values may result in the assay of penicillin by any method unless it is shown that the total antibacterial activity of the sample is due exclusively to true penicillin. This means first establishing that true penicillin is present and that it is the only antibacterial substance present. The significance of this point cannot be overemphasized since it is now known that cultures of *Penicillium notatum* may contain at least two antibacterial substances (Kocholaty, 1942; Coulthard, *et al.*, 1942; Waksman and Woodruff, 1942; Roberts, *et al.*, 1943). One of these, notatin (Coulthard, *et al.*, 1942) [also called coli factor (Waksman and Woodruff, 1942), penatin (Kocholaty, 1942), and penicillin B (Roberts, *et al.*, 1943)] has antibacterial activity only in the presence of glucose. The assay medium for penicillin contains no glucose, but culture filtrates of the mold invariably contain residual carbohydrate, and the amount incorporated into the assay medium with the sample being tested often is sufficient for the action of notatin.

The solubility of penicillin in organic solvents makes it important to consider the combined effect of solvent and penicillin. Thus, table 1, part A, shows the lethal concentrations of various solvents on the growth of *Staphylococcus aureus* H under the assay conditions (plate method, see below). Part B shows that sub-inhibitory doses of solvents have an additive effect on penicillin which renders the penicillin potency apparently higher than it actually is. This discrepancy is great enough to yield misleading results. It is obvious that penicillin samples for assay should be in aqueous solution containing, if possible, no solvents.

Designations of activity

Numerous terms have been used to express penicillin activity. These include the dilution, the number of micrograms and the number of Florey units causing inhibition.

The dilution terminology, while a more traditional means of expression, has a serious disadvantage in that where a high degree of accuracy is required, the values obtained are not sufficiently rigorous to be accepted without reservation,

TABLE 1
*Influence of solvents on penicillin assay**
Part A. Effect of solvents alone

	ML. SOLVENT PER 10 ML. AGAR		
	0.033	0.1	0.3
Amyl acetate	+	+	—
Ethyl acetate	0.1	0.33	1.0
Acetone	+	+	—
Chloroform	0.1	0.33	1.0
Ethyl alcohol	±	—	—
NaHCO ₃ (sat. sol'n.)	0.063	0.2	0.6
Ether	+	+	+
	0.1	0.33	1.0
	+	+	+

Part B. Effect of solvents with penicillin

SOLVENT PER 10 ML. AGAR	PENICILLIN† (MICROGRAMS PER 10 ML. AGAR)						
	0	8	10	12	14	16	17
No solvent	+	+	+	+	+	±	—
0.05 ml. amyl acetate	+	±	+	+	—	—	—
0.10 ml. amyl acetate	+	±	—	±	—	—	—
0.05 ml. chloroform	+	+	+	±	±	—	—
0.10 ml. chloroform	+	—	—	—	—	—	—

* +, growth; —, complete inhibition.

† Containing 6 Florey units per mgm.

due chiefly to variables in the assay. These may cause a significant deviation from day to day in the value of the same sample. Also, dilution results express only a range of values between which the real endpoint lies (see below).

Standard penicillin preparations

To eliminate the day-to-day deviation, the Oxford group introduced the concept of the penicillin unit and the use of a standard penicillin preparation. The potency of any penicillin sample is obtained by direct comparison with the primary standard or indirectly against secondary standards whose potencies

have been previously established by the primary Oxford standard. First called the Oxford unit, it has lately come to be known as the Florey unit after Professor Florey of Oxford University. Use of a standard run daily, side by side with the unknown test solutions, minimizes variations caused by slight changes in media, condition of and amount of inoculum, incubation time, etc., since the standards and unknowns presumably are affected alike.

Although the Oxford group explained the origin of the penicillin unit, it is not permissible for other laboratories to establish in the same way the unitage of their own preparations. The idea of Florey unitage today connotes a *comparison either directly or indirectly against the original Oxford standard of known unitage*. On account of the deviations caused by uncontrollable factors, attempts to evaluate the potency of samples after the manner of the Oxford definition give at best only an approximate value since the Florey unit applies *specifically and only* to the original standard set apart by the British workers and designated by them to contain so many units per milligram. The fact that they named the unit that amount¹ of penicillin which under the conditions of the cup assay gives an inhibition zone 24 mm. in diameter does not mean that every laboratory will arrive at the same value for the unit, even though each follows faithfully the definition laid down by Abraham, *et al.* (1941). From the experience in this laboratory values obtained were found to vary considerably from the true Florey unit. This laboratory will be glad to supply, without cost, small quantities of secondary penicillin standards which have been accurately standardized indirectly against the authentic Oxford standard.

Preparation of standards

Because of the tendency of some penicillin samples to deteriorate rapidly upon standing, the first requisite of a preparation to be chosen as a standard is that it shall be of *proven stability*. The criteria for the stability are obvious; the selected samples must be shown to have lost no activity during storage over a considerable time. In these laboratories, fairly large samples (1 to 2 grams) of four different penicillin preparations are stored in dry ice for a period of not less than two months, during which time they are tested once or twice each week. If they show no loss of activity, they are acceptable as standards. Dry ice storage is maintained as an extra precaution for stability. Refrigerator temperatures probably are satisfactory. Besides establishing stability, the weekly assays over a 2- to 3-month period serve to give a Florey unitage which is the average of several assays on different days and on different weighed samples. Table 2 shows the data establishing the stability and potency of a secondary standard. The primary standard in this case was obtained from Oxford.

Test bacteria

For the sake of uniformity and standardization, it would seem most desirable to employ the strain of *Staphylococcus aureus* H originally used by the Oxford

¹ Originally, this amount, when dissolved in 50 ml. of meat extract broth just inhibited completely the growth of the test strain of *S. aureus*. Thus, material containing one unit per mgm. just inhibits the growth of *S. aureus* at 1:50,000.

TABLE 2
Potency and stability of a secondary standard

SOLUTION FOR ASSAY MADE UP ON	DATE OF ASSAY ON SOLUTION	ASSAYED BY	METHOD	FLOREY UNITS PER MCM.
4/28/42	4/28	B. L. W.	Turbidimetric	61.7
	4/28	B. L. W.	Turbidimetric	59.8
	4/30	B. L. W.	Turbidimetric	74
	4/30	N. G. H.	Cup	48
5/4	5/4	B. L. W.	Turbidimetric	85*
	5/4	B. L. W.	Turbidimetric	69.5
	5/5	B. L. W.	Turbidimetric	52.5
5/12	5/12	B. L. W.	Turbidimetric	63.2
	5/12	H. B. W.	Cup	58
5/12	5/20	H. B. W.	Cup	59
	5/20	H. B. W.	Cup	60
6/22	6/22	B. L. W.	Turbidimetric	61.6
	6/22	B. L. W.	Turbidimetric	50
	6/22	H. B. W.	Cup	62
	6/23	H. B. W.	Cup	56
6/24	6/24	H. B. W.	Cup	46.8
	6/25	H. B. W.	Cup	61
	6/25	H. B. W.	Cup	67
	6/25	B. L. W.	Turbidimetric	68
	6/25	B. L. W.	Turbidimetric	61.7
	6/25	B. L. W.	Turbidimetric	60
	6/24	H. B. W.	Cup	64.2
	6/25	H. B. W.	Cup	81.5
6/29	6/25	H. B. W.	Cup	66.0
	6/29	B. L. W.	Turbidimetric	56.2
	6/29	B. L. W.	Turbidimetric	58.6
	6/29	B. L. W.	Turbidimetric	58.6
	7/2	H. B. W.	Turbidimetric	55.4
	7/2	H. B. W.	Turbidimetric	61.7
	7/2	H. B. W.	Turbidimetric	63.0

Average values

SAMPLE	AVERAGE OF INDIVIDUAL ASSAYS ON EACH SOLUTION	REMARKS
4/28	60.9	Value of 85 excluded from averages
5/4	61.0	
5/12	60.6	
5/20	59.5	
6/22	60.8	
6/29	58.9	
Grand average.....	60.3	

* Obviously too high, discarded.

group to assay penicillin. Subcultures are available from these laboratories upon request. Use of a penicillin standard tends to reduce the importance of the authentic *S. aureus* H culture; however, when a standard is not employed,

it is imperative to use the H strain. It is virtually meaningless to use any strain of *S. aureus* for a quantitative method because different strains may vary markedly in their sensitivity. This was already noted for certain other bacteria in the Abraham, *et al.* paper (1941). Table 3 compares the sensitivities of a number of different strains of *S. aureus* and stresses the desirability of using a standard strain for penicillin assays.

Tables 4 and 5 bring out the variations in the sensitivity of *S. aureus* H when assayed at different times and on different media. It is obvious that a significant variation in sensitivity may occur from time to time and from medium to medium. The only explanation at present is the empirical claim for "variation".

TABLE 3
Sensitivity of different strains of S. aureus

Figures represent Florey units per ml. of brain-heart agar causing inhibition of a streak of the organism. Where two figures are given, the first did not inhibit and the second did.

STRAIN	FLOREY UNITS
1	0.0096-0.108
2	.024 - .030
3	.030
4	.024
5	.030
6	.010 - .012
7	.030 - .060
8	.60
9	.03 - .10
10	.03 - .10
11	.01 - .03

The following four strains were tested in liquid nutrient broth by the dilution method:

STRAIN	INHIBITING DILUTION OF PENICILLIN BROTH
A	50
B	166-250
C	166-250
D	50-100

Further to minimize the variation due to condition of inoculum, the following procedure for handling the test organism has been adopted. Stock nutrient agar slants are stored in a refrigerator. At monthly intervals or so, subculture slants are made from them, which, in turn, are kept cold after overnight growth at 37°C. Overnight broth cultures are made each day from a slant, the same slant being kept cold and used for about a week. Serial transfer in broth has at times led to abnormalities such as development of resistant cells and of cells subject to lysis during penicillin inhibition.

Individual methods of assay

Serial dilution. This procedure consists of adding by suitable dilution procedures different amounts of penicillin to either liquid or solid media.

a. Liquid broth assays: Fleming, in 1929, first utilized the serial dilution procedure for penicillin, and many workers consider it, or modifications thereof the most useful for routine assays. But again it is important to specify the order of accuracy required. As pointed out above, without a penicillin standard the results cannot be comparable to those of other laboratories unless the standard strain of *S. aureus* H is employed as the test organism. Even then the results may be taken only as an order of magnitude of potency owing to uncontrollable daily fluctuations. The precision of this method depends on the steps in dilution, and in our experience a clear-cut plus and minus endpoint occurs only where the dilutions differ by a factor of at least 50%. This accuracy is tolerable only in those cases where real quantitative results are not the main object.

Sterile samples and aseptic technique are required. In many cases the samples must be Seitz-filtered before use. With high potency samples the high

TABLE 4

Variations in sensitivity of S. aureus H on different media

Figures represent Florey units per ml. of agar causing inhibition of a streak of the organism. Where two figures are given, the first did not inhibit and the second did.

DATE	MEDIUM	FLOREY UNITS
7/15/42	Blood agar	0.005-.0075
9/ 3/42	Brain-heart	0.014-.024
10/ 8/42	Nutrient	0.10 -.30
10/ 8/42	Brain-heart	0.010-.030
10/13/42	Brain-heart	0.010-.030
10/13/42	Nutrient	0.030
11/12/42	Brain-heart	0.013-.014
1/ 6/43	Yeast-extract glucose	0.090-.012
1/ 6/43	Yeast-extract	0.015

dilution required to reach the endpoint usually eliminates contaminations. With small amounts of material filtration losses may be significant, either mechanically or possibly through adsorption. In general, Seitz filtration may be accomplished without loss of potency. Often samples which are too contaminated for use directly may be pasteurized (60°C. for 30 minutes). However, a definite though variable deterioration usually results from pasteurization, ranging from 5 to 30 per cent of the activity.

Daily employment of a penicillin standard puts the dilution assay on a sound basis, but, nevertheless, the factor of poor sensitivity is not eliminated. As mentioned previously, this requirement depends on the experimental objectives. Steps in dilution less than 100 per cent as a rule give progressive degrees of inhibition which make a decision as to the real endpoint difficult. Furthermore, on penicillin samples of unknown potency, use of smaller steps in dilution necessitates an excessive number of dilutions to insure the end-point falling within the range selected. The alternative would be to cover a wide range with larger intervals between dilutions, thereby establishing the approximate value, and

then, on a second assay, run it down within a limited range. In any case, extra work is involved, and where large numbers of assays are run routinely, such procedures are excessive. The act of inoculating each dilution tube separately is itself an undesirable routine. The endpoint obtained by this method may be anywhere between that dilution which inhibits completely and that which does not. For many kinds of chemical experiments on penicillin a specific value is desirable and such accuracy is inadequate.

Rammelkamp's (1942) modification of the tube dilution method is designed to meet the requirements of the clinician who is faced with the problem of having only small amounts of blood or body fluid available for assay. Very low levels of penicillin, such as would be contained in a small amount of blood must be measured. This was achieved with a strain of *Streptococcus hemolyticus*, selected

TABLE 5
Variability in daily plate assays on a sample of penicillin
(This solution contained 0.24 mg. crude penicillin per ml.)

DATE ASSAYED	ML PER 10 ML AGAR REQUIRED TO INHIBIT <i>S. AUREUS</i>
5/14/42	0.001
5/15	.0009
5/16	.0009
5/18	.0011
5/19	less than .0008
5/20	greater than .0011
5/21	.0009-.001
5/22	less than .0008
5/23	.0011
5/25	less than .0008
5/26	.0011
5/27	greater than .0011
5/28	.001-.002

because of its high sensitivity to penicillin. Thus, levels and amounts of penicillin as low as 0.0039 Florey unit in 0.2 ml. [see also (Foster, 1942)] can be measured in this way by comparison with a standard. The sensitivity undoubtedly could be further increased with a more susceptible test organism. The presence of 1% erythrocytes in the veal infusion medium facilitates reading the endpoint resulting through hemolysis accompanying growth. Although this method permits a useful sensitivity, its accuracy is good only to 100 per cent since the dilutions differ by factors of 2, and in certain cases only partial inhibition is obtained. While undoubtedly of value to the clinician, this order of accuracy is quite unsuited for chemical studies.

Through the courtesy of Dr. J. C. Hoogerheide of the Squibb Biological Laboratories, the details of a modified broth dilution method devised in those laboratories are available. An accuracy of 15 per cent is stated. Two ml. amounts of beef-heart infusion broth containing 0.25% glucose and inoculated with a 1 to 10 dilution of a 6-hour culture of *S. aureus* H are distributed in a

series of ten small tubes. To one tube each is added respectively with a micro pipette, 0.10, .09, .08, .07, .06, .05, .045, .040, .035, and .030 ml. of a standard made up to contain 0.60 Florey units per ml. Similar amounts of the unknowns diluted to 0.60 units per ml. on the basis of predicted potency are added to other series of tubes. After overnight incubation, the endpoint is stated to be reproducibly clear-cut between two tubes. The endpoint is not necessarily that dilution which inhibits growth completely but, rather, that dilution in which growth is so retarded that the organism fails to develop a uniform turbidity throughout the broth, forming, instead, a sediment in the bottom of the tube leaving a clear supernatant. The endpoint on the unknown contains that amount of penicillin corresponding to the endpoint in the standard series. Samples would have to be reasonably free from contaminating bacteria. Aside from the tedium of making a large number of accurately measured pipettings, this would appear to be one of the most promising methods for practical application.

b. Plate assays: Since these are serial dilution methods, the same comments outlined above on expression of activities and numbers of dilutions required also apply here. Different amounts of the appropriate dilution of penicillin are mixed with 10 ml. of melted agar in plates, and after solidification, the surface of the plate is streaked with the test organism and the point of complete inhibition obtained after overnight incubation. An incidental advantage here is that as many as six different test organisms may be tested on each plate, i.e., at each dilution, but this is unnecessary for routine assays. One feature of dubious advantage is that fairly heavily contaminated samples can still be read, but this loses importance because even lightly contaminated samples, especially in solution form, can be partially inactivated by penicillase-producing bacterial contaminants (Abraham and Chain, 1940).

The cooled melted (nutrient or brain-heart) agar may be seeded with the test organism before distributing the agar, thereby eliminating streaking. About 0.1 ml. of a 20 hour nutrient broth culture is used per 100 ml. agar. Plates remaining clear after overnight incubation have inhibited the test organism. While the vast majority of cells may be inhibited, a few isolated colonies persistently develop over two or three successive dilutions. These resistant colonies usually are insignificant in numbers and may be disregarded for practical purposes. In many instances the inhibition is quite gradual in a series of close dilutions, making the endpoint difficult to establish.

Plate methods, requiring numbers of plates and large volumes of agar, are at best very tedious and on account of the disadvantages mentioned above are not to be preferred. A standard is imperative for best accuracy. The variability of results obtained from day to day on a stable sample without a standard is exemplified in table 5.

The results show the frequency of "greater than" or "less than" values, which, of course, require repetition of the assay. Where two values are given, partial inhibition was observed over that range.

The pH of the assay medium has an unexpected effect on penicillin activity in

the plate method. Nutrient agar was adjusted in lots to pH 5.5, 6.0, 6.5, and 7.0, respectively, and the plate assay run as described with *S. aureus* H. Table 6 shows that the activity of penicillin is increased with lowered pH at least down to pH 5.5 and that a difference of 0.5 pH unit definitely influences the results. A sensitivity effect at pH 5.5 greater than 3-fold that at 7.0 was obtained in this experiment. The same type of effect was noted with other sensitive bacteria, including *S. hemolyticus*, *Micrococcus lysodeikticus*, *Salmonella paratyphi* A, and *Staphylococcus albus*. The interpretation of this effect is not clear at present. Indeed, when this experiment was repeated in liquid media with the turbidimetric method discussed below, no significant difference in penicillin action at the various pH levels could be detected. The details of this experiment together with a discussion will be given in the next paper of this series.

Turbidimetric method. This method has already been described by this laboratory (Foster, 1942), and the details will not be repeated here. In our experience it is the most accurate method available, but to attain this high

TABLE 6
Effect of pH of test medium on inhibition of S. aureus in plate assay

PENICILLIN (MICROGRAMS PER ML. AGAR)	pH			
	5.5	6.0	6.5	7.0
0	+	+	+	+
.08	—	+	+	+
.12	—	—	+	+
.16	—	—	±	+
.20	—	—	—	+
.24	—	—	—	±

+, growth; —, inhibition.

precision, the special and fastidious techniques required render it rather cumbersome for the handling of many assays. An accuracy of ± 10 to 15 per cent is obtainable. For special purposes, such as, for example, in stability experiments, molecular weight studies by diffusion, etc., it has proved most valuable.

The method is based on the proportional inhibition of growth of *S. aureus* H in liquid media as a function of penicillin concentration. Growth measurements are made with a photoelectric colorimeter. A standard curve is run daily with the unknowns, which themselves are run at 3 to 5 different dilutions, depending on how many can be predicted to fall on the central three-quarters region of the standard curve. Potencies of the unknowns are computed after comparison with the standard curve which covers accurately about an 8-fold range in values. Rigid observance of technique, accuracy, and the need for aseptic precautions detract from its usefulness as a general method. In addition, operations attendant to reading the turbidities are time-consuming.

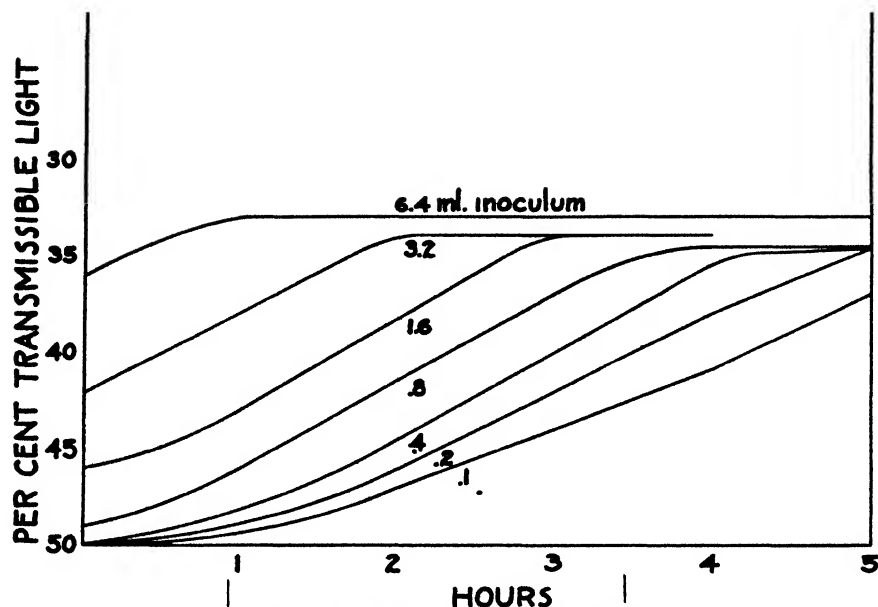
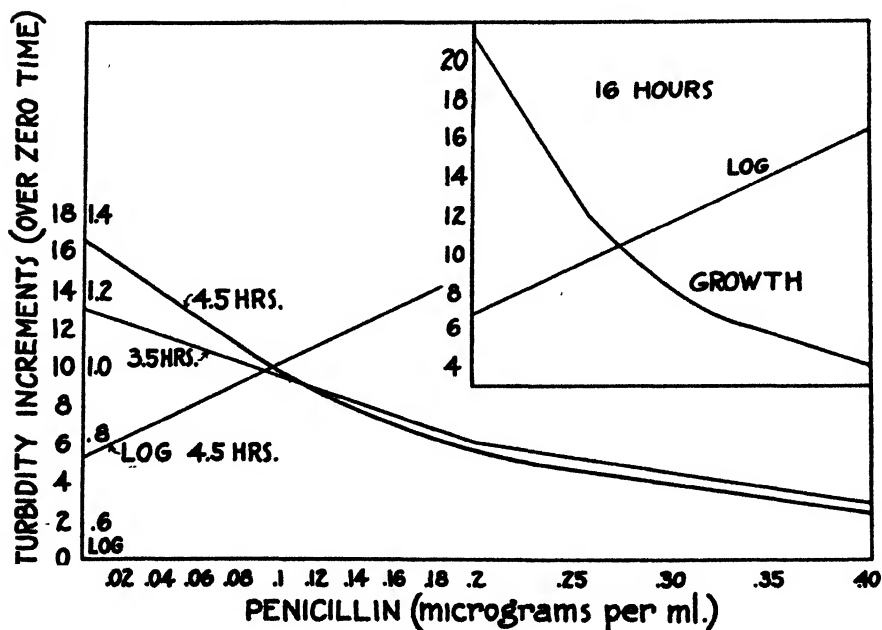
Turbidimetry offers a means of securing results in a time shorter than the 16 or 20 hours required at present. For routine work little is gained by setting up 20 to 50 assays and having the results in the same working day since probably little further experimental work could be done that day on the basis of those

results. In certain instances, however, a real need exists for a short time assay. For example, it is crucial to determine when a large scale production batch of penicillin is ready for extraction. Maximum accumulation of penicillin in cultures of *Penicillium notatum* frequently persists for only a brief period, after which the potency may drop off sharply. To know the strength of a production batch within a few hours of the time of sampling would aid greatly in eliminating such losses. The time required for the assay can, theoretically, be reduced by increasing the sensitivity of the method used to measure growth. With the sensitivity that the regular Evelyn photoelectric colorimeter affords, a satisfactory growth spread can be obtained in about 4 hours by accelerating the rate of growth by use of a heavy inoculum. Figure 1 shows the effect of different amounts of a 20 hour nutrient broth culture as inoculum on the growth curve of *S. aureus* hourly for 5 hours. The lag phase seems appreciable only below 0.4 ml. inoculum, and the maximum rate of growth is constant for this and the higher levels. In 4 hours the 0.4 ml. inoculum made the greatest increase in turbidity at a constant rate and, hence, is the most suitable level for an assay of 4 hours' duration. Figure 2 shows the penicillin inhibition in nutrient broth after a short time. The inset shows the type of standard curve obtained in 16 hours' incubation. Only the first half of the short time curves are useful for assay purposes. Beyond that, the turbidity change with increments of penicillin is too small. Before reading short time turbidities, all tubes are placed in cold water to stop growth. Samples that have been assayed by the short time method agree with the regular overnight method, but results generally are less reproducible in the former. Nevertheless, this method has found valuable application in our laboratories.

Results obtained turbidimetrically check well with values obtained by the Oxford cup method. The following list compares the values on 7 samples:

SAMPLE	FLOREY UNITS PER MG.		PERCENTAGE DIFFERENCE
	Turbidimetric	Cup	
A	0.97	0.90	+10
B	15.4	14.7	+5
C	7.3	7.0	+4
D	9.7	11.2	-15
E	11.0	13.2	-17
F	26.0	26.4	-2
G	17.4	17.2	+1

Oxford cup method. Except for certain modifications, the published details (Abraham, *et al.*, 1941) of this procedure will not be repeated here. Small porcelain or glass tubes set on the surface of solidified inoculated agar plates are filled with penicillin solutions and incubated. Where the penicillin diffuses out into the agar, growth of the test organism is inhibited and a circular clear zone results. Within limits, the diameter of the zone is a function of penicillin concentration, and the unknowns can be calculated in terms of the standard curve of inhibition which is drawn for each day's run.

FIG. 1. INOCULUM SIZE AND *S. AUREUS* H GROWTHFIG. 2. PENICILLIN INHIBITION OF *S. AUREUS* WITH TIME

This penicillin contained 10 Florey units per mgm. Medium: nutrient broth.

In our experience the cup method is by far the most useful routine assay method. One person can assay at least twice as many samples with the cup method as by any other tried. If the potency of the sample can be predicted

approximately, only one dilution is required for assay; otherwise 2 and at most 3 would be needed. The standard curve covers about a 7-fold range (0.2 to 1.5 Florey units per ml.). A specific value is always obtained instead of the range characteristic of dilution methods. We average the quadruplicate values obtained on the final dilution predicted to contain one Florey unit per ml. At some sacrifice in accuracy, only two or three replicates may be run, but this accuracy probably would be well within the range of that of dilution methods. Our experience substantiates the ± 25 per cent accuracy claimed for the method (Abraham *et al.*, 1941). In practice, the work involved in running extra replicates is practically negligible. Most laboratories which employ this method concur in regard to its simplicity and general usefulness. The procedure is not nearly so cumbersome as other methods.

The unwieldy procedure of inoculation described originally by the Oxford workers can be effectively replaced by seeding the cooled, melted agar before pouring the plates. One-tenth ml. of a 24-hour nutrient broth culture is used to seed 100 ml. of cooled, melted agar. The agar should be uniformly measured into each plate by apportioning 13 ml. amounts with an open tip pipette.

The sharp bevelled edge of the cups recommended by the Oxford group to obtain a seal between the tube and the surface of the agar is unnecessary. Difficulties in obtaining such bevelled tubes has tended to discourage their use. Uniform length tubes made from 9 mm. outside diameter (1 mm. wall thickness) glass tubing with a glass cutting machine work just as satisfactorily if, just before planting on the agar, one end of the tube is passed momentarily through a flame to warm it slightly. It melts the agar locally, which sets again practically immediately, making a good seal. This step requires very little extra time in setting up the plates for use. The tubes can be from 6 to 12 mm. in length, but those employed on any one assay run must all be of uniform size, and they must be filled uniformly to the top with the solution.

Serious irregularities encountered with large numbers of assays revealed that if as little as one hour elapses between the times that the standard penicillin solutions and unknown samples are put into the cups, a significant discrepancy in values occurs. Handling a large number of assays is time-consuming, and no matter when the standard is set up, some of the samples are likely to be set up and incubated two or more hours before or after. The following experiment illustrates this effect. The cups in seeded plates were filled with portions of one solution at four hourly intervals. All plates were left on the laboratory bench until the last one was completed and then incubated at 37°C. overnight. The results are listed as follows:

TIME OF ASSAY	AVERAGE DIAMETER OF INHIBITION ZONES	FLOREY UNITS	PER CENT DIFFERENCES FROM TRUE VALUE (4:00 P.M.)
	mm.		
1:00 p.m.	24.8	1.28	68
2:00	23.5	0.97	27
3:00	23.0	0.88	16
4:00	22.4	0.76	—

Thus, plates allowed to stand at room temperature for 1, 2 and 3 hours before incubation give proportionately higher values than the sample incubated at 37°C. immediately after filling. The differences are large enough to be serious. The explanation lies in the fact that during the intervals of 3, 2 and 1 hour, respectively, the penicillin diffuses out into the surrounding agar. Meanwhile, at room temperature the bacterial cells seeded in the agar make very little, if any, growth, but when placed at 37°C., the growth is rapid. Those plates standing at room temperature show larger zones because the penicillin has a longer time to diffuse before growth appears. In the case of incubation directly at 37°C., growth appears before diffusion is complete, hence the smaller zones. For uniform results it is apparent that the cells should make no growth from the time the agar is poured until the plates are placed at 37°C. Possibly this could be achieved by keeping the plates cold until diffusion is complete; this is being tested. To standardize diffusion with respect to growth, as short a time as possible should elapse between filling the cups and incubating at 37°C. Thus, there is an equal period of growth during the active diffusion for all plates from the time the samples are added. In our laboratories, four plates, each containing six cups, are handled at a time. These accommodate six samples on each plate. Plates and dilutions are prepared in advance and stored cold as below; filling the cups in four plates takes about ten minutes after which each set of plates is immediately placed at 37°C. When assaying low potency *Penicillium* filtrates extreme pH's of the samples should be neutralized.

To have a number of poured seeded plates on hand at one time and to prevent growth until the plates are used, a large number of seeded plates are made and stored in a refrigerator and withdrawn for use four at a time. This idea was tested by assaying a sample in 18 replicates on seeded plates that had been stored in a refrigerator for 0, 1.5 and 23 hours. The large number of replicates was taken to give an indication of the normal variation in zone size. The standard was run at zero time.

HOURS IN REFRIGERATOR	DIAMETER OF INHIBITION ZONES (MM.)						AVERAGE ZONE SIZE	FLOREY UNITS PER MCM. OF SAMPLE	DIFFERENCES FROM ZERO TIME
							mm		per cent
0	28.1	28.0	27.5	27.8	28.0	27.5	28.0	144	
	28.5	28.0	28.0	28.0	27.5	28.0			
	28.0	28.2	27.5	28.0	28.0	28.0			
1½	27.5	28.0	28.1	28.5	28.0	28.2	28.1	147	2
	29.0	28.0	28.3	27.3	29.0	28.0			
	28.0	28.0	28.0	28.0	28.1	27.5			
23	29.0	29.0	27.3	27.0	29.0	29.0	28.2	150	4
	28.5	28.2	28.2	27.5	28.0	28.0			
	28.0	29.0	28.6	27.7	27.5	29.0			

It is evident that so long as growth is not permitted to commence until just after filling the cups, the seeded plates may be stored at refrigerator temperature at

least as long as 23 hours without influencing the assay results. The variation between replicates is small, but it is important to have at least two replicate cups, and preferably more. The best average value is obtained by having each cup of each sample on different plates.

One practiced person can conveniently assay in quadruplicate cups about 40 to 50 samples per day. Readings are facilitated by placing the Petri dish with zones over an exposed photographic plate with fine millimeter lines cut into the gelatin. This is set over an opening in a block with a diffuse lighting source shining through. The size of the zones is defined by the white lines against the dark background.

Through the kindness of Dr. A. H. Dowdy of the Strong Memorial Hospital, University of Rochester, we are able to reveal an unpublished novel simplification of the cup method which Helen and James Vincent of that institution have developed in collaboration with Dr. Dowdy. Instead of using cups, discs of filter paper saturated with the penicillin samples are used to produce the zones of inhibition. This feature should reduce materially the labor involved in setting up large numbers of assays, and, therefore, ultimately may find valuable application.

Miscellaneous Assay Methods

A number of different principles have been tested for possible use in measuring penicillin and especially for short time assays. For the most part, unpromising results have been obtained; in some cases the preparation and special technique are not conveniently adaptable. Following are some of the ideas tried without success.

1. Methylene-blue reduction by washed cells of a susceptible organism.
2. Inhibition of luminescence in cultures of luminescent bacteria.
3. Microscopic observation of cessation of motility of bacteria.
4. Microscopic observation of appearance of enlarged and involution cell shapes.
5. Titration of acid formed by lactic acid bacteria, *S. aureus*, etc. This may have application for overnight assay.

SUMMARY

The principles, merits and disadvantages of the different bacteriological methods used for the quantitative determination of penicillin are summarized and discussed. The numbers of assays to be run, the accuracy desired, and the facilities available govern the final choice of a method. A standard penicillin preparation of known Florey unitage to be run daily with unknowns is recommended to minimize uncontrollable fluctuations. Serial dilution methods, with liquid or solid media, are wanting in accuracy and do not yield specific values for penicillin activity. A modified broth method devised by the Squibb laboratories gives good sensitivity and is practical. The turbidimetric method has application in special types of investigations where high accuracy is required but is too fastidious for routine assays. The Oxford cup method (modified) is

an excellent all-around method for routine assays, especially for large numbers. Its accuracy is good, a specific value is obtained, and, from the standpoint of labor involved, it is quite the most rapid procedure where accuracy is required. A number of experiments characterizing the different methods are reported.

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THE COAGULATION AND STERILIZATION OF CULTURE MEDIA

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Methods recommended for the coagulation and sterilization of blood serum or egg media by the use of steam under pressure are of two types. In the first, all, or some indefinite part, of the air originally present is retained in the autoclave by keeping all valves closed except the steam inlet during both coagulation and sterilization. This results in temperatures below the maximum attainable for the operating pressure and in variations of temperature in different parts of the autoclave due to stratification of the steam; the indefinite temperature established makes prolonged treatment necessary; and, in the case of autoclaves which have their thermometers in the exhaust, the temperature cannot be ascertained. In the second type, the air is retained in the autoclave during the coagulation period and then is permitted to escape slowly, avoiding any pressure drop until all the air has been replaced by steam. The sterilization period follows with the temperature in the autoclave then at the maximum attainable for the operating pressure.

The available directions for the second method are most ambiguous and contradictory. It is not generally recognized that the preparation of coagulated and 121°C. sterilized egg media is less of a problem than the preparation of blood serum media. The same directions are commonly given for both types. (A.P.H.A. 1941) Egg media (Petragnani's and an egg yolk medium, McCarter and Kanne, 1942) were satisfactorily prepared and sterilized at 120°C. in both a modern, leak-free, single jacketed autoclave and in a very old, very leaky, single jacketed autoclave. The autoclaves were characterized by the fact that, with all valves closed after a pressure of 15 pounds was established in the air-free autoclave, the pressure dropped to zero pounds (atmospheric pressure) within 21 minutes in the better of the two autoclaves, and within 4½ minutes in the leaky autoclave. The better autoclave was used to produce satisfactory egg yolk medium without taking any but the usual precautions. That is, a wooden platform or shelf was used in the autoclave to prevent conduction of heat from the autoclave to the tubes of medium contained in an ordinary bacteriological metal test tube rack, and all valves were closed at the end of the sterilization period. The same satisfactory medium was prepared in the very leaky autoclave by degassing the medium before dispensing it into tubes and autoclaving. The degassing was accomplished by boiling the medium under vacuum at from room temperature to 45°C. The same medium, run as a control and not degassed, was completely disrupted in the leaky autoclave. The time for the pressure to drop from 15 pounds to zero pounds was kept the same in both autoclaves by gradually cutting off the steam supply. A batch of Petragnani's medium was prepared

satisfactorily in both autoclaves only if previously degassed, not quite as satisfactorily in the better autoclave if not first degassed, and completely disrupted in the old autoclave if not first degassed. It was thereby indicated that if egg media were protected against heat conducted from the autoclave walls by using a wooden shelf, the chief reason for failure to prepare satisfactory media of these types was the presence of gases in the medium. This was most strikingly evident when the medium was tubed in such volume as to form both a slanted surface and a butt in the tube. The food canner uses a similar degassing procedure to minimize changes in oxygen labile ingredients when foods and fruit juices are heat processed. (Ayers, 1938) There is no readily available evidence of analogous research in the preparation of culture media.

The sterilization of Loeffler's blood serum mixture at 121°C. was not made possible by the above method. There was some, but little, gas retained in this medium when prepared from the Difco (1939) dehydrated product. (There may be considerable gas present if an old hydrated batch of this dehydrated medium is used.) This medium was apparently more difficult to sterilize at 121°C. than were the egg media because blood serum coagula either boil or synerize objectionably at temperature-pressure combinations more similar to those characteristic of steam under pressure, that is, at approximately those temperatures at which water boils at various pressures. Blood-serum media sterilized at 121°C. and at the corresponding pressure of 15 pounds therefore should not be permitted to cool more slowly than the steam under pressure in the autoclave. Such a disparity in rate of cooling and rate of pressure drop was, finally, very simply established without pumping air into the autoclave after the sterilization period. This was done by placing into the autoclave a completely closed, empty, wooden (swamp cyprus) box, equal to approximately one-fifth of the capacity of the autoclave. This empty box served as the insulating platform upon which the metal racks of tubed media rested; it served as a reservoir, or pocket, of air and steam (leaking through the joints in the box) which did not prevent the establishment of the maximum temperature possible for the operating pressure; it did not prevent the continuous flow of steam into and out of the autoclave during the sterilization period; it permitted continuous temperature indication on the thermometer in the steam exhaust line; and it approximately doubled the time (21 to 38 minutes) required for the pressure to drop from 15 pounds to zero pounds after having closed all valves at the end of the sterilization period. In this way satisfactory slants and butts of the medium were prepared.

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BACTERIAL MORPHOLOGY AS SHOWN BY THE ELECTRON MICROSCOPE

VI. CAPSULE, CELL-WALL AND INNER PROTOPLASM OF PNEUMOCOCCUS, TYPE III

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Electron micrographs of the bacterial cell-wall and inner protoplasm, intra- and extra-cellular granules, spores and flagella have been presented in earlier papers in this journal and elsewhere (Mudd and Anderson, 1941, 1942; Mudd, Polevitzky and Anderson, 1942, 1943; Knaysi and Mudd, 1943). The pneumococcal capsule is technically a more difficult subject, primarily because it scatters the electron beam so little as to afford very poor contrast. With care the pneumococcal capsule can be demonstrated in electron pictures, however, as a definite structure of low density surrounding the cell-wall, the cell-wall in turn is demonstrable in this as in other bacteria as a distinct structure surrounding the inner protoplasm with its limiting protoplasmic membrane (Knaysi, 1938, 1941). Visualization of capsule, cell-wall and inner protoplasm as separate and distinct morphological entities, we hope may prove clarifying.

The technic which has proved most satisfactory for preparing pneumococci for the electron microscope is exceedingly simple: a clean inoculating loop is dipped into distilled water. A clean inoculating needle is touched to the surface of a pneumococcal colony and then thrust repeatedly through the film of water held in the inoculating loop; the film is then touched to the collodion mount; the resulting droplet on the mount is allowed to dry. The preparation may be introduced directly into the microscope; or the preparation may be washed by dipping repeatedly into distilled water and again dried; or a drop of serum dilution or chemical reagent may be placed on the specimen and after a suitable interval removed by dipping into distilled water. Pictures were taken with the 60-kilowatt commercial model RCA electron microscope, usually at an electronic magnification on the plate of 7,000 diameters; prints were subsequently enlarged to the desired optical magnification.

The pneumococci were grown for 18 to 20 hours on 5% horse-blood infusion agar plates incubated in large air-tight jars to preserve moisture.

Figure 1 shows the capsule surrounding cells of pneumococcus, Type III. The capsule appears, in the words of Pasteur's (1881) original description, as "*une sorte d'auréole*." Extraneous granules on the mount are visible through the

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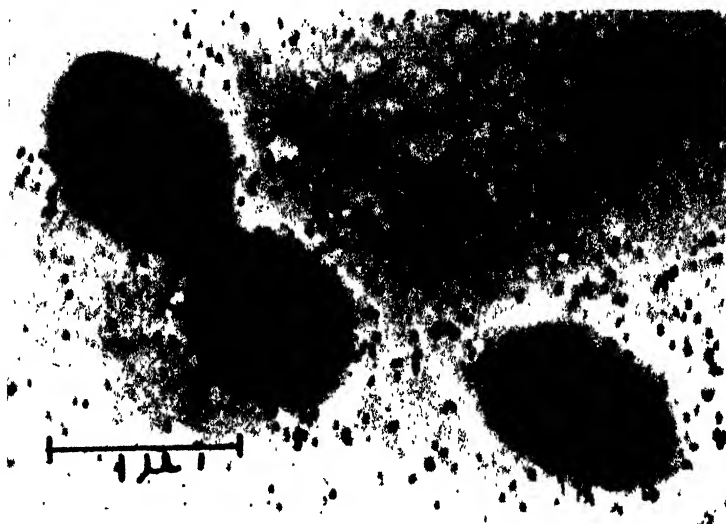


FIG. 1. PNEUMOCOCCUS, TYPE III

The capsule appears as an aureole surrounding the cell-walls. $\times 25,000$



FIG. 2. NON-ENCAPSULATED PNEUMOCOCCI (ROCKEFELLER INSTITUTE STRAIN DERIVED FROM TYPE I)

The inner protoplasm has shrunk away from the cell-walls. $\times 13,000$

capsule.² This picture, in printing, has been slightly over-developed with respect to the pneumococcal cell in order to make the aureole visible at all.

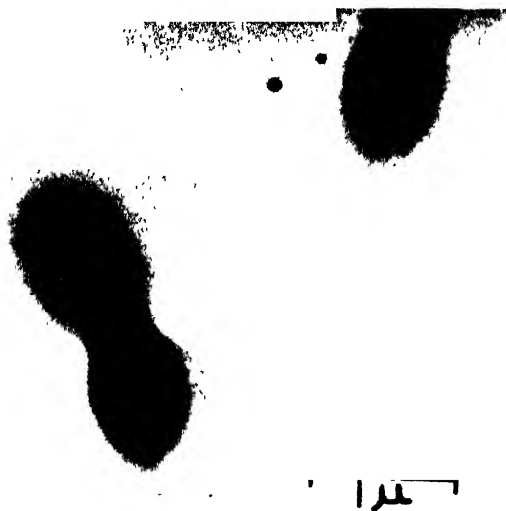


FIG. 3. NON ENCAPSULATED PNEUMOCOCCI AS IN FIGURE 2. $\times 18,700$

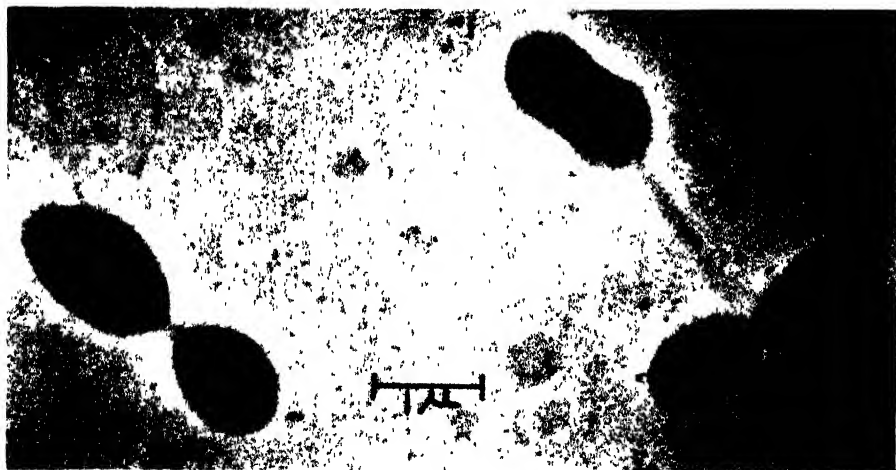


FIG. 4. PNEUMOCOCCI, TYPE III—CELLS WASHED IN 0.1 N NaCl SOLUTION, THEN IN DISTILLED WATER

A delicate strand of protoplasm still connects the diplococcal pair at the right. A strand continuous with the cell-walls connects the diplococcal pair to the left. The capsular aureole surrounds all the cocci. $\times 14,000$.

Figures 2 and 3 show, for comparison, cells of a non-encapsulated variant. These pictures were not overdeveloped; nevertheless the cell-walls, from which

² Under present conditions the depth of focus of the electron microscope is of the order of 5μ (Hillier, 1943), so that the image of the entire thickness of the preparation is projected onto the photographic plate.

the inner protoplasm has shrunk away, appear more definite and less transparent than the capsules in figure 1.

Figure 4 shows diplococci in three stages of division. The uppermost pair is in an early stage; the pair immediately below shows only a delicate strand of protoplasm connecting the cells of the diplococcus; the third pair are united only by a strand continuous with the cell-walls. A narrow aureole of capsular material surrounds each diplococcus.

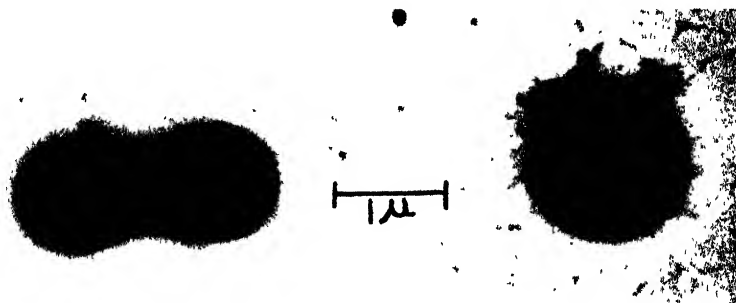


FIG. 5. PNEUMOCOCCUS, TYPE III. UNWASHED CAPSULATED CELLS SURROUNDED BY EXCESS OF SECRETION PRODUCT. $\times 14,000$

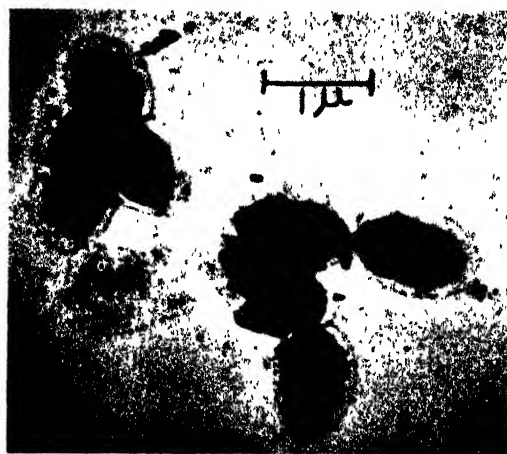


FIG. 6. PNEUMOCOCCUS, TYPE III, AFTER EXPOSURE FOR 30 SECONDS TO M 7 LEAD ACETATE SOLUTION. $\times 14,000$

The term "capsule," according to definitions proposed by Ettinger-Tuleczynska (1933) which we believe to be of value, should be reserved for a relatively definite and characteristic structure immediately outside the cell-wall; the pneumococcal capsule of course corresponds to this definition. Extra-cellular mucoid material which is relatively indefinite and inconstant, on the other hand, is called by Ettinger-Tuleczynska a "Schleimhülle." According to this author pneumococcus Type III may possess both capsule and slime-layer. Figure 5 illustrates capsulated pneumococcal Type III cells mounted and dried for micrography without washing. A "slime-layer" of excess secretion product obviously surrounds the capsulated bacterial cells.

Somewhat characteristic effects of salts of heavy metals upon the protoplasm of non-capsulated bacteria have been described in an earlier publication (Mudd and Anderson, 1942). In view of the report by Etlinger-Tuleczynska (1933) that the pneumococcal capsule may be stained without "swelling" by certain metal salts, we have exposed preparations of Type III pneumococcus to solutions of several salts of heavy metals. A few pictures of interest have been obtained.

Figure 6 shows cells of pneumococcus Type III after exposure for 30 seconds to a M/7 solution of lead acetate. The peripheries of the capsules show definitely increased density, suggesting the deposition there of lead.

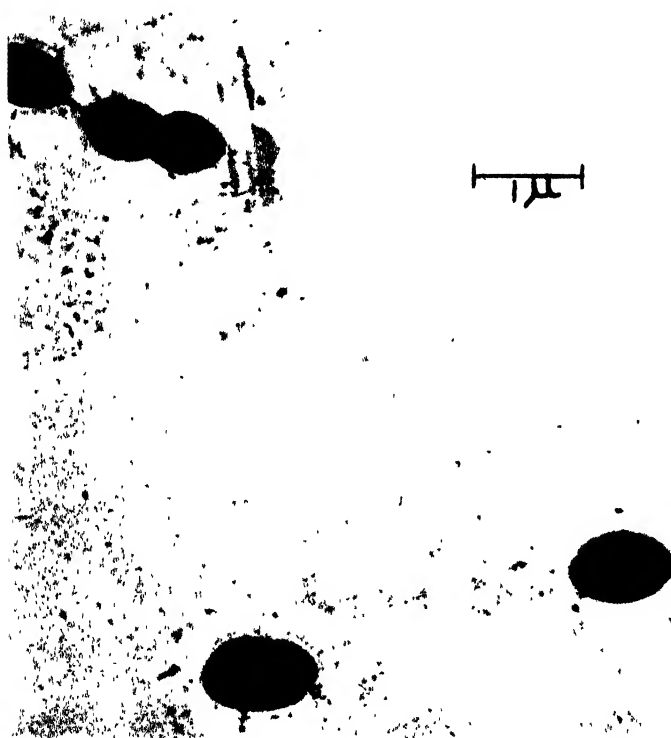


FIG. 7. PNEUMOCOCCUS, TYPE III, AFTER EXPOSURE FOR 30 SECONDS TO SATURATED COPPER SULFATE SOLUTION. $\times 14,000$

Figure 7 shows a similar, although slightly less definite, effect of saturated copper sulfate. In the cell at the bottom of figure 7 the dark protoplasm has shrunk away from the cell-wall and the delicate outline of the capsule is visible outside the cell-wall.

Treatment of pneumococcal cells with M/15 aluminum sulfate has removed the capsule except for a residue of delicate fibrils radiating from the cell-walls; marked shrinkage effects of the protoplasm from the cell-walls were also obtained. Such a preparation of pneumococcus Type I is shown in figure 8; similar effects have been obtained with M/15 $\text{Al}_2(\text{SO}_4)_3$ and Type III cells, but we have not so good a picture of Type III.

In a study of the pneumococcal capsular swelling phenomenon (Mudd, Heinmets and Anderson, 1943), it is shown that specific antibody and nonspecific serum components permeate the interstices of the capsule with resulting increase in thickness and density. The capsular carbohydrate, when prepared with scrupulous precautions against alteration (Heidelberger, Kendall and Scherp, 1936), has been found to be in the form of thread-shaped carbohydrate polymers. From these several data the conclusion seems justified that the pneumococcal capsule is a highly hydrated gel of low density.

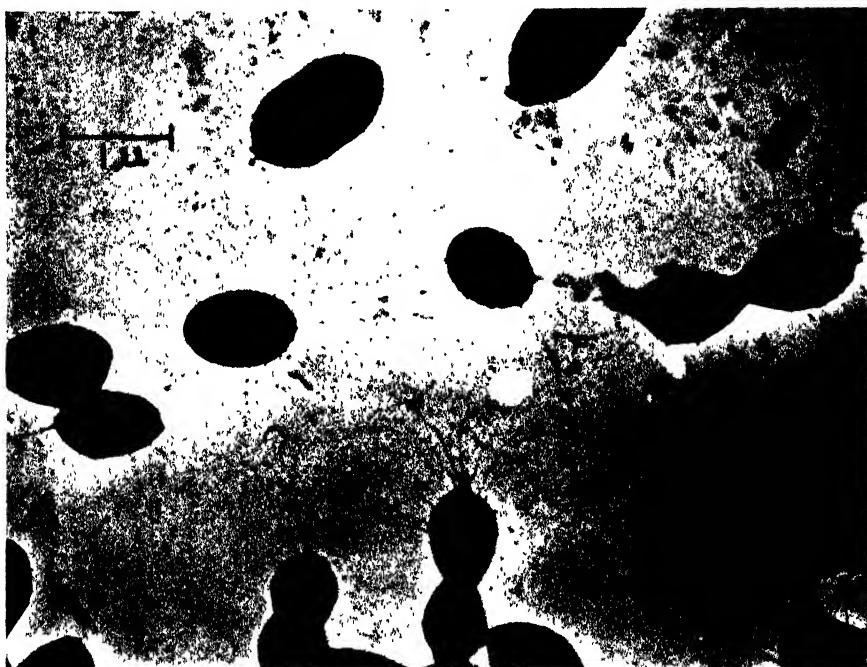


FIG. 8. PNEUMOCOCCUS, TYPE I, AFTER EXPOSURE FOR 30 SECONDS TO M/15 ALUMINUM SULFATE SOLUTION
Similar effects were obtained with Type III cells. $\times 14,000$

CONCLUSION

The pneumococcal capsule is a gel of low density immediately surrounding the cell-wall. The cell-wall, in turn, is distinct from the inner bacterial protoplasm with its limiting protoplasmic membrane.

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NOTES

A NOTE ON THE TAXONOMY OF *PROTEUS HYDROPHILUS*¹

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Two recent reports (Kulp and Borden, 1942; Guthrie and Hitchner, 1943) have shown that the organism known as *Proteus hydrophilus* is polarly flagellated. Hence this species must be removed from the *Enterobacteriaceae* and placed in the *Pseudomonadaceae*.

Kulp and Borden have also confirmed the finding of previous workers that *P. hydrophilus* ferments carbohydrates with vigorous gas production. This is surprising in view of the extreme rarity of true fermentative ability (*sensu* Pasteur) in the *Pseudomonadaceae*; in fact, the only previous well-established example is the alcoholic fermentation which was shown by Kluyver and Hoppenbrouwers (1931) to be characteristic of *Pseudomonas lindneri*. Hence a more detailed study of the action of *P. hydrophilus* on sugars seemed desirable. An examination of several strains obtained through the kindness of Drs. Kulp and Hitchner has revealed that *P. hydrophilus* carries out a typical butylene-glycol fermentation substantially identical with that of *Aerobacter aerogenes*.

The outstanding fermentative properties of *P. hydrophilus* thus clearly call for a generic separation from other polarly flagellated rods. The appropriate genus, *Aeromonas*, has already been proposed by Kluyver and van Niel (1936) for Beijerinck's (1900) polarly flagellated *Aerobacter liquefaciens*. Although authentic cultures are not available for comparison, it is highly probable that *Aerobacter liquefaciens* is identical with *P. hydrophilus*. Indeed, an extensive synonymy appears to have grown up around *P. hydrophilus*. Guthrie and Hitchner have suggested its identity with *Pseudomonas punctata*, originally described by Zimmermann (1890); it is also indistinguishable culturally and biochemically from *Pseudomonas fermentans* (von Wolzogen Kühr, 1932). Since these organisms were isolated from water and not tested for pathogenicity, it is readily understandable that their relationships to so vigorous a disease-producer as *P. hydrophilus* should have been overlooked. The correct name for *Proteus hydrophilus* is thus *Aeromonas hydrophila*, although if its suggested synonymy with *Pseudomonas punctata* is proved, the latter specific name will have priority.

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ON THE USE OF HYDROLYZED WHEAT MASH FOR THE ENRICHMENT OF CLOSTRIDIUM ACETOBUTYLICUM¹

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Rubbo *et al.* (1941) suggested a wheat-mash medium for the selective cultivation of *Clostridium acetobutylicum*. Most other anaerobes and the sporing aerobes failed to grow on the medium when anaerobic conditions were provided. The constant need for new strains of this organism for the biological production of acetone and butyl alcohol on industrial scale suggested immediately a possible use of the medium as a selective enrichment medium.

Following exactly the technique of Rubbo *et al.*, with the exception of the use of American grains, media were prepared from the following: wheat, cracked wheat, yellow corn and white corn. These media were tested for their ability to support the growth of authentic strains of *Clostridium acetobutylicum*, *C. roseum*, *C. felsineum*, and three butyric-acid-producing clostridia. *C. roseum* and *C. felsineum* were included because of their close affinity to *C. acetobutylicum* in physiological reactions. The results were not encouraging. All types of media either failed to support growth or did so only when the inoculum was relatively large—0.2 to 0.5 ml. of an active culture. Usually, if the medium supported growth of *C. acetobutylicum*, other cultures, including butyrics, also developed. In view of the negative results several different batches of the media were tested and these were prepared by different individuals to eliminate, if possible, personal factors in the technique. These all failed to be selective for *C. acetobutylicum*. The media were tested by seeding plates and/or deep columns in tubes, or both. Yeast-water glucose agar, included as a control, gave consistently positive cultures with smaller inocula than those which were positive with the hydrolyzed mash media. The pH determinations were made with a Beckman electrode. All plates were incubated in an oat jar (McClung,

¹ This problem has been supported, in part, by a grant from the Research Fund of the Graduate School of Indiana University.

McCoy and Fred, 1935) and negative results were discarded unless yeast-water glucose agar plates within each jar were positive.

On the basis of the above results it is concluded that the hydrolyzed wheat medium, prepared from American grains, may not prove to be a successful enrichment medium for *C. acetobutylicum*.

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USE OF DRIED TISSUE IN BEEF HEART MEDIUM FOR ANAEROBIC BACTERIA¹

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Although the thioglycollate medium of Brewer (1940) has been shown to be satisfactory for the enrichment of *Clostridium welchii* and other spore-forming anaerobes (McClung, 1940, 1943; Marshall, Gunnison and Luxen, 1940 and others), it may not replace entirely the time-honored beef-heart infusion medium, due to unavailability of the dehydrated medium in certain regions or personal preferences of some investigators. The common practice of preparing each batch of beef-heart medium from fresh tissue has many obvious disadvantages. Following an observation by W. G. Roessler, in these laboratories, that the tissue could be dried and used in a medium destined for routine transfer of certain proteolytic anaerobes, a short study has been made of the possibilities of this technique. The results show that it is possible to dry the tissue after extraction and use it with the broth (sterilized and stored in screw-capped bottles) at a later time, as needed, to prepare a medium comparable to that made from the tissue before drying. No unusual equipment is needed and the convenience of the technique is suggested to those using beef-heart medium in civilian or military clinical laboratories.

The medium we have tested was prepared according to the method previously published (McClung, 1940). In these experiments the tissue was squeezed dry by hand pressure, spread in a thin layer on two thicknesses of cheese cloth, and

¹ This problem has been supported, in part, by a grant from the Research Fund of the Graduate School of Indiana University.

placed on a wire shelf directly over the heating element of a 37°C. incubator room. As an aid in rapid dehydration a stream of air was blown across the tissue by a small fan. We used the furnace blower (model # 581 C 7550) of the Montgomery Ward Company; doubtless any other small fan would serve as well. The dehydration is accomplished in a few hours by this system. Earlier studies revealed the need for the provision of rapid circulation of the warm air.

Comparable cultural reactions were obtained when a large series of different species were tested in media prepared from fresh and from dried tissue. The latter had been stored for one month at room temperature. Furthermore, the two types of media gave almost identical results in ability to initiate growth from inocula containing a small number of cells when tested with *Clostridium welchii*, *C. septicum*, *C. oedematiens*, *C. tertium*, *C. histolyticum*, *C. sporogenes*, *C. botulinum*, and *C. tetani*.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

WASHINGTON BRANCH

ONE HUNDRED AND THIRTY-SECOND MEETING, APRIL 27, 1943

STUDIES ON STRAINS OF *AEROBACTER CLOACAE* RESPONSIBLE FOR ACUTE ILLNESS AMONG WORKERS USING LOW-GRADE STAINED COTTON. *B. H. Caminita, R. Schneiter, R. W. Kolb, and P. A. Neal*, Division of Industrial Hygiene, National Institute of Health, U. S. Public Health Service, Bethesda, Maryland.

A gram-negative, motile, mucoid, non-sporulating, rod-shaped microorganism was found to occur in large numbers in low-grade, stained cotton, which caused illness among workers; the same organism was also found in large numbers in dust from cotton mills, hemp mills, and grain elevators. Workers exposed to hemp dust or grain dust are known to suffer from an illness similar to that described in workers in low-grade cotton.

An intensive study was made of 107 cultures isolated from samples of raw cotton, cotton seed, cotton mill dust, elevator dust screenings, hemp mill dust, and retted hemp plants, and from cases of illness.

The organisms were slow-lactose-fermenters, their Imvic reaction was $--++$; most of the cultures isolated actively attacked the usual carbohydrate test substances except adonitol, inositol, and inulin; they produced only acid in glycerol and liquefied gelatin; they gave a characteristic heavy mucoid growth on glucose agar; and under suitable conditions most of them produced yellow pigment. According to Bergey's Manual, fifth edition, the Imvic reaction, gelatin liquefaction, and lack of active glycerol fermentation are characteristic of *Aerobacter cloacae* as distinguished from *Aerobacter aerogenes*. The organisms referred to in previous papers as the "cotton bacterium" were therefore classified as *A. cloacae*. There did not appear to be sufficient difference between the strains studied and cultures of *A. cloacae* obtained for comparison from the American Type Culture

Collection to justify classifying these strains as a new variety of *A. cloacae*.

Four types were arbitrarily differentiated on the basis of biochemical tests: Type 1 produced yellow pigment and fermented dulcitol and sorbitol but not inositol; Type 2 was white and fermented sorbitol but not inositol or dulcitol; Type 3 was white or yellow, fermented inositol but usually not dulcitol and sorbitol; Type 4 varied widely in fermentation reactions on the various carbohydrates. Types 1, 2, and 3 fermented saccharides rapidly with reversion of the reaction of the medium.

Type 1 cultures produced an endotoxin capable of causing illness in human beings when inhaled, although the bacteria themselves did not appear to survive longer than 48 hours in the human respiratory tract. They had a low pathogenicity for laboratory animals. Small doses of the endotoxin injected intravenously killed rabbits but not mice or chickens.

Antibodies could be produced in rabbits by intravenous injections of either the endotoxic substance or killed cultures. The cultures tested against rabbit immune sera appeared to be heterologous although a considerable degree of cross agglutination appeared in low dilutions.

The organisms were not pathogenic when inoculated into cotton seedlings, but were saprophytic in immature cotton bolls into which they were introduced.

RETENTION OF SULFONAMIDE RESISTANCE AND INCREASED p-AMINO-BENZOIC ACID PRODUCTION IN *STAPHYLOCOCCUS AUREUS*. *Maurice Landy and Ruth B. Gerstung*, Division of Bacteriology, Army Medical School.

STREPTOCOCCIC BACTERIOPHAGE: A REVIEW. *Alice C. Evans*, National Institute of Health.

THE OCCURRENCE AND BACTERIOLOGICAL CHARACTERISTICS OF *S. MARCESCENS* FROM A CASE OF MENINGITIS. *Joseph D. Aronson and Ilo Alderman*, Station Hospital, Fort Belvoir, Va.

From the spinal fluid of a colored soldier there was obtained on two different occasions a profuse growth of grayish-pink colonies which assumed deep red color when exposed to daylight. Microscopically, small, motile, gram-negative organisms were found. The cultural and biochemical characteristics were those of *Serratia marcescens*. The culture was highly pathogenic for white mice, white rats, hamsters, turtles and rabbits, but not for guinea pigs. The pigment from this culture extracted in ace-

tone, petroleum ether, amyl alcohol, xylene, decinormal hydrochloric acid, water, benzene, chloroform, carbon tetrachloride, and carbon disulphide, showed a maximum absorption of wavelengths ranging from 532-550. Ether, ethyl, and methyl alcohol extracts showed an absorption of wavelengths in the 460 and 536 zone while the decinormal sodium hydroxide extract showed maximum absorption in the 494, 536, 572 wavelengths. Bacterial dissociation manifested itself by the appearance of nonchromogenic colonies and by variations in the colonial characteristics. No significant differences were noted in the virulence of the nonchromogenic and chromogenic colonies.

THE TEXAS BRANCH

DALLAS, TEXAS, MAY 8, 1943

PROTECTIVE EFFECT OF SEPARATE INOCULATION OF SPOTTED FEVER VIRUS AND IMMUNE SERUM BY INTRADERMAL ROUTE. *Ludwik Anigstein, Madero N. Bader, and Gerald Young*, Dep't. Preventive Medicine and Public Health, The U. of T. Medical School, Galveston.

The purpose of the present work is to investigate the possibility of protecting a susceptible animal against spotted fever by using small amounts of specific rabbit hyper-immune serum. The intradermal route of inoculating guinea pigs with virus and serum was used.

Three main factors were considered: the site of inoculation, the time factor, and the amount of immune-serum.

An area of $\frac{1}{4}$ square inch of guinea pig's skin was infiltrated with 0.4 ml. of immune serum and two hours later 0.1 ml. of virus was injected into this area. These animals remained afebrile and proved immune to subsequent reinfection with spotted fever.

Fifteen guinea pigs were then inoculated simultaneously at the same site with 0.1 ml. of virus and 0.4 ml. immune serum. No fever was noted in eleven of them, while four developed spotted fever. Both groups showed solid immunity to subsequent reinfection with spotted fever. Six control guinea pigs were injected with virus and serum as above but at different sites of the body, and five developed typical spotted

fever. In the next series smaller amounts of immune serum were used: 0.1 ml., 0.05 ml. and 0.025 ml., the injections were given simultaneously and at the same site with the virus. Out of 13 guinea pigs none developed spotted fever. Four weeks later they were reinfected with a five-fold dose of virus and nine animals showed complete immunity.

Experiments are now under way to find out the lapse of time subsequent to virus inoculation after which immune serum will still protect the animal. Inactivation of the virus in the skin was followed by solid immunity even when 0.025 ml. of immune serum was injected at the same site as much as 18 hours following the virus injection.

It will be of interest to investigate whether it is possible to prevent the disease by applying the above method after infectious tick-bite.

FIELD AND LABORATORY STUDIES OF POLIO-MYELITIS IN TEXAS. *J. V. Irons and S. W. Bohls*, Bureau of Laboratories, Texas State Department of Health, Austin, Texas.

Until late in October, 1942, the incidence of poliomyelitis in Texas had been slightly lower than usual; but in the late fall and early winter the incidence was above expectations, particularly in some South Texas communities. Evidence of multiple infections was found in certain families.

By the inoculation of young Swiss mice and cotton rats with specimens of brain, spinal fluid, blood, or stool, we failed to obtain or maintain the causative virus.

Seventy-five per cent of human sera examined appeared to have neutralizing activity against the Armstrong poliomyelitis virus, but the percentage was lower with specimens from young children. Sera from children in acute stage of poliomyelitis have most frequently lacked evidence of neutralizing antibodies. We have failed with the few human sera from recognized cases, carefully followed to date, to obtain significant increases in neutralizing titers against the Armstrong virus, possibly, in part at least, because of difficult maintenance of sufficiently high titratable potency of virus suspensions.

Except for an exposure to certain arthropods and rodents, no common factor was found in all cases. Pools of collected, identified arthropods have been stored in dry ice for later tests on monkeys.

VARIATION OF BRUCELLA SPP. WITH REFERENCE TO THE BACTERIOSTATIC ACTION OF DYES. *Gordon Worley, Jr. and Jane Read Worley*, Brucellosis Research Project, Clayton Foundation, The University of Texas, Austin.

This report deals with one of the standard procedures employed in the differentiation of the species *Brucella*, namely the bacteriostatic action of dyes. In appropriate concentrations of the dyes *Brucella suis* is inhibited by basic fuchsin, *Brucella abortus* by thionin, while *Brucella melitensis* is inhibited by neither dye.

Using the technique employed by I. M. Lewis in his work on *Escherichia coli-mutabile*, we placed into a sterile Petri dish a massive inoculum of *Brucella suis* (all the growth from a tryptose agar slant) and then added tryptose agar containing basic fuchsin in an amount usually inhibitory for *B. suis*. After incubation, the plates showed the appearance of a few colonies which had grown quite readily. When these colonies were fished to tryptose agar, the resultant culture had the ability to grow luxuriantly on basic fuchsin agar, a characteristic lacking in the parent strain. Such cultures,

then, gave reactions on the dyes which were identical with those of *Brucella melitensis*.

With three different *Brucella suis* stock strains, we were able to obtain such variants from each. When these variants were carried for ten serial transplants on tryptose agar, they still were able to grow on the fuchsin medium. The fuchsin variants also had a decidedly increased tolerance for the dye pyronine.

The variant cultures could not be distinguished from the parent strains by microscopic appearance, by fermentation reactions, or by colonial appearance on tryptose agar.

As yet no variants have been obtained from *Brucella abortus* with thionin as the test dye.

PRELIMINARY OBSERVATIONS OF GROWTH OF SELECTED STRAINS OF BRUCELLA IN THE McCULLOUGH AND DICK MINERAL BASE MEDIUM. *V. T. Schuhardt and Grace A. Beal*, Brucellosis Research Project, Clayton Foundation, The University of Texas, Austin.

Two strains each of *Brucella abortus*, *Brucella melitensis* and *Brucella suis* were tested for their requirements of the four growth factors, thiamin, niacin, biotin and pantothenic acid, using the McCullough and Dick mineral base medium with $(\text{NH}_4)_2\text{SO}_4$ as the only source of nitrogen and glucose the only source of carbon. One strain of *B. melitensis* failed to grow in the presence of all four growth factors whereas the other five strains of *Brucella* gave uniform growth through ten transfers when all four factors were present. The strain of *B. melitensis* which grew and the two strains of *B. suis* required the presence of thiamin and niacin. The strains of *B. abortus* required the presence of biotin in addition to thiamin and niacin. These growth factor requirements are the same as those reported by Koser *et al.* who used an amino-acid base medium.

A SURVEY OF TRYPANOSOMA CRUZI INFECTION IN TRIATOMA SPP. COLLECTED IN TEXAS. *Thelma deShazo*, Bureau of Laboratories, Texas State Department of Health, Austin, Texas.

Seven hundred and four *Triatomae* have been examined for *Trypanosoma cruzi* infection at the Texas State Department of Health Laboratory from July 19, 1941 through November 14, 1942. Eight species of *Triatoma* were included in the survey. These were collected from twenty-four counties in Texas and included *T. gerstaeckeri* (30.91% positive); *T. heidemannii* (59.86% positive); *T. sanguisuga* (19.23% positive); *T. protracta* (34.37% positive); *T. ambigua* (45.45% positive); *T. occulta* (50% positive); *T. neotomae* (87.5% positive); and *T. rubida* (100% positive). A total of 36.5% were found to be naturally infected with *T. cruzi*.

Little difference was noted in the positive ratio between male (43.37%) and female (49.47%) adults. However, a significant difference occurred in the positive nymph-adult ratio; only 27.1% of the nymphs were positive as compared with 47.81% positive adults.

Triatoma neotomae has not been reported previously as naturally infected with *T. cruzi*.

RECENT OBSERVATIONS ON RELAPSING FEVER. Donald V. Moore and D. C. Thurman, Bureau of Laboratories, Texas State Department of Health, Austin, Texas. Observations have been made on a strain

of spirochetes found in *Ornithodoros talaje*. To date no significant differences have been noted between this strain of spirochetes and the strain transmitted by *Ornithodoros turicata* except that attempts to infect guinea pigs with the *talaje* strain have been unsuccessful.

A brief description of the laboratory diagnosis of relapsing fever is given. The comparative effectiveness of the stained thick smear and darkfield examinations of laboratory animals was studied. 173 such examinations were chosen. In the stained blood smears 88 of these 173 examinations were negative and 85 positive. Of the darkfield examinations made at the same time the blood smears were taken 147 were negative and 26 positive. These figures show that 49% of the stained smears were positive; while only 15% of the darkfield examinations were positive.

Records of human cases of relapsing fever in Texas were discussed. Five new counties were added to the existing list of counties in Texas where human cases of relapsing fever have been known to exist.

Observations on a laboratory infection of relapsing fever were discussed.

THE LABORATORY IN THE DIAGNOSIS OF VIRUS DISEASES. S. E. Sulkin, St. Louis Dept. Health, St. Louis, Missouri.

CENTRAL NEW YORK BRANCH

NEW YORK STATE AGRICULTURAL EXPERIMENT STATION, GENEVA, NEW YORK,
MAY 15, 1943

THE SANITATION OF PYREX GLASS TUBING USED TO REPLACE METAL TUBING IN FOOD AND DAIRY PLANTS. G. J. Hucker, N. Y. State Agricultural Experiment Station, Geneva.

A CHEMICALLY DEFINED MEDIUM FOR THE CULTIVATION OF THE GONOCOCCUS. Jane Plack, H. E. Stokinger, and C. M. Carpenter. University of Rochester, Rochester.

A medium comprising 9 organic acids, 5 inorganic salts and glucose has been developed for the growth of the gonococcus. It has the following composition in grams per liter: *d*-glutamic acid, 1.3; *dl*-leucine, 0.40; *d*-arginine monohydrochloride, 0.25;

d-histidine monohydrochloride, 0.15; *dl*-methionine, 0.15; *L*-proline, 0.10; glycine, 0.05; *L*-cystine, 0.01; indole-3-acetic acid, 0.10; NaCl, 6.0; NaH₂PO₄·H₂O, 2.5; NH₄Cl, 1.25; Mg(NO₃)₂·6H₂O, 0.05; FeSO₄, 0.012; and glucose, 5.0.

Sterile solutions of glucose, magnesium nitrate, and ferrous sulfate are added separately to a solution of the remaining constituents at pH 7.0 previously autoclaved at 121°C for 10 minutes. The pH is adjusted to 7.2.

The medium was inoculated with washed gonococcal cells obtained by centrifugation from a 24-hour Douglas's broth culture and incubated at 37°C. in an atmosphere containing approximately 10 per cent of carbon

dioxide. Growth of stock strains of the gonococcus was maximal after 2-3 days. 0.25 mg. of bacterial nitrogen, equivalent to 2.0 mg. of cells, was obtained for each 5.0 ml. of medium. The cells remained viable for at least 5 days when the gaseous mixture was replaced daily. Recently isolated strains grew better in the medium than in Douglas's broth, but not as well as in blood broth. Cultures transferred every third day were maintained for 3 months, and would doubtless grow indefinitely.

PREPARATION OF SPECIFIC POLYSACCHARIDE FROM TYPE I MENINGOCOCCI GROWN IN A CHEMICALLY DEFINED MEDIUM. *Henry W. Scherp*, University of Rochester, Rochester.

Specific polysaccharide may be prepared from cultures of Type I meningococci in Frantz' medium. Because of the large proportion of acid produced, the phosphate buffer is increased to 0.05 molar, omitting an amount of sodium chloride equivalent to the increase. Growth in relatively thin layers is essential (e.g., 500 ml. of medium in 2000 ml. Florence flasks). Carbon dioxide is not required.

A virulent strain of the organism is cultured in embryonated eggs, and transferred to "starter" cultures in the Frantz medium, from which in turn mass cultures are inoculated. After 14 days at 37°C., the cultures are processed through the following steps: twenty-fold concentration *in vacuo*; removal of the constituents of the medium by dialysis; removal of most of the protein by iso-electric precipitation at pH 4.8; removal of the remainder by treatment with cupric acetate at pH 5.0; repeated precipitation of the polysaccharide by 5 volumes of absolute ethyl alcohol, from solution in normal sodium-acetate-acetic acid buffer of pH 4.0. The yield varies from 1.15 to 0.16 g. per 10 liters of medium. Immunological activity is uniformly high, as little as 0.15 μ g. giving an immediate precipitin test with antimeningococcal horse serum.

PRODUCTION OF ACID FROM GLUCOSE AND MALTOSE BY TYPE I MENINGOCOCCI IN A CHEMICALLY DEFINED MEDIUM. *Henry W. Scherp*, University of Rochester, Rochester.

THE ROLE OF pH IN DISINFECTION AND ANTISEPSIS. *Otto Rahn and Jean E. Conn*, College of Agriculture, Cornell University, Ithaca.

BACTERIOSTASIS BY CRYSTAL VIOLET. *C. E. Hoffmann*, College of Agriculture, Cornell University, Ithaca.

PRACTICAL VALUES FROM TAXONOMIC RESEARCH. *Robert S. Breed*, New York State Experiment Station, Geneva.

Recent studies that will be reported in the sixth edition of the Bergey Manual reveal many instances where previously unsuspected relationships have been discovered by taxonomic research. The placing of *Serratia* in the family *Enterobacteriaceae* has emphasized the existence of yellow, golden brown and red strains of *Escherichia*. Serological studies have supported the close relationship of these genera. Likewise, the placing of *Erwinia* in *Enterobacteriaceae* has met approval. In this case it is the soft rot organisms (*E. carotovora*, etc.) that are most closely related to the coliform organisms. Current research has shown that the organism causing "red leg" of frogs (*Proteus hydrophilus*) is perhaps identical with *Pseudomonas punctata*, the organism which causes a septicemia of carp.

The clarification of the nature of *Cytophaga* and *Sporocytophaga* and their position among the slime bacteria emphasizes the part these organisms play in nature in bringing about the decomposition of plant materials.

The suggestion of a relationship between the organisms known as *Zoogloea ramigera* and bacteria found in activated sludge should stimulate comparisons between these and *Nitrosogloea* and *Nitrogloea* H. Winogradsky.

Additional instances might be cited where taxonomic research has proved productive. Competent workers, not amateurs, are needed in this field.

ACIDITY AND HYDROGEN-ION CONCENTRATION RELATIONSHIPS AMONG THE LACTIC ACID BACTERIA. *Carl S. Pederson and Josephine V. Bagg*, New York State Experiment Station, Geneva.

Characterization of lactic acid bacteria is

dependent upon the utilization of various carbon compounds. Criteria for utilization commonly employed are either change in acidity or in hydrogen-ion concentration.

Due to variables involved, total acidity and hydrogen-ion concentration relationships as produced by growth of bacteria are complex. In milk, the most highly buffered medium used, the highest acidity and lowest hydrogen-ion concentration was obtained. Higher acidity was produced from pentose sugars than from hexose sugars with less change in hydrogen-ion concentration. Likewise, non-gas-producing lactic acid bacteria caused greater changes in hydrogen-ion concentration than gas-producing strains with the same amount of titrable acid. These differences are due to the relative amount of lactic and acetic acids produced. Higher total titrable acidity may be obtained from the pentose sugars than from hexose sugars because larger amounts of acetic acid are produced and acetic acid changes the hydrogen-ion concentration less.

Final hydrogen-ion concentration is a limiting factor but not the only limiting factor for fermentation. With low total acidity, the magnitude of the change in hydrogen-ion concentration is greater than the change in total acidity, but with higher acidity, total acidity changes are greater.

VIABILITY OF *STREPTOCOCCUS LACTIS*. H. B. Naylor, College of Agriculture, Cornell University, Ithaca.

SEPARATING SOIL ALGAE FROM OTHER ORGANISMS BY MEANS OF THE CENTRIFUGE. J. K. Wilson and P. J. Westgate, New York State College of Agriculture, Cornell University, Ithaca.

Algae, natively present in the soil, have been successfully separated by centrifugation from other organisms. The enriched culture was developed in light in a mineral solution, and then centrifuged at 1800 r.p.m. for one minute. The supernatant liquid was decanted, leaving the algae in the tubes, sterile water added, and the algal growth dispersed. This washing was repeated six times. The algal cells were distributed in Petri dishes by the dilution method. An agar medium containing only nutrient salts was used. The plates were placed under a 40-watt light for a period of approximately fourteen days, at which time green colonies usually appeared. Such colonies were transferred to agar slopes and tested for purity by growing them on a medium containing sucrose and beef extract, and by subsequent examination under the microscope. By careful selection of colonies many pure cultures of algae were obtained.

THE RESULTS OF SOME EXPERIMENTS WHERE SALT WAS ADDED IN SILAGE MAKING. J. K. Wilson, Cornell University, Ithaca.

THE NATURE AND MODE OF ACTION OF ANTI-BIOTIC SUBSTANCES. Selman Waksman, New Jersey Agricultural Experiment Station, New Brunswick, New Jersey.

FIFTH MEETING NORTHERN-CALIFORNIA HAWAIIAN BRANCH

UNIVERSITY OF CALIFORNIA, BERKELEY, JUNE 5, 1943

STUDIES ON THE NATURE AND CONTROL OF DISSOCIATION IN *BRUCELLA ABORTUS*. Werner Braun, Division of Veterinary Science University of California, Berkeley.

Attempts are being made to investigate the nature and cause of dissociation in *Bruceella abortus* by an analysis of the chemical changes involved in this process.

The effect on dissociation of ultra-violet rays of different wave length, urea solutions, and ribonuclease has been investigated (see

J. Bact., 45: 21, 1943). Limited results suggest that the effectiveness of such agents in producing dissociation and reducing viability may differ for different strains and this possible difference is being investigated. Differences in amino-acids and vitamins in the variants are being analyzed with the help of new micro-biological tests. The Feulgen reaction, specific for thymonucleic acid, was applied to bacterial suspensions, using a high-speed centrifuge for the stain-

ing procedure; resulting color differences are being measured with the help of a photometer. Urea which produces dissociation in the test tube experiment is being studied for its effect on *Brucella abortus* within the animal body.

In order to classify the variants a comparative scale has been developed utilizing ground glass of different grain, slightly tinted with transparent paint; also a rapid "microagglutination" test has been perfected by suspending cells in drops of 1:2000 fuchsin solution on depression slides for 30 minutes at 37°C.

STUDIES ON THE NATURE AND CONTROL OF DISSOCIATION IN *BRUCELLA ABORTUS*. *W. Braun*, University of California.

PHYSIOLOGY AND NUTRITIONAL REQUIREMENTS OF *PASTEURELLA PESTIS*. *M. Doudoroff*, University of California.

MICROBIAL TARTRATE DECOMPOSITION. *R. Vaughan*, University of California.

AGGLUTININS IN SWINE BLOOD SERUM FOLLOWING THE FEEDING OF BOVINE FETAL AND PLACENTAL TISSUE INFECTED WITH *BRUCELLA ABORTUS*. *C. M. Haring*, University of California.

RAPID PRODUCTION OF PENICILLIN. *C. E. Clifton*, Stanford University.

THE EFFECT OF VARIOUS SUBSTANCES ON THE OXIDATION OF CERTAIN SUGARS AND PYRUVIC ACID BY A TYPE I AVIRULENT PNEUMOCOCCUS

FREDERICK BERNHEIM AND MARY L. C. BERNHEIM

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Sevag (1933) studied the oxidation of glucose, lactic and pyruvic acids by various pneumococci. During the oxidation of glucose and of lactic acid, hydrogen peroxide is formed which eventually inhibits the oxidation of these substrates. The hydrogen peroxide can be removed either by the addition of catalase or by the addition of pyruvic acid which acts as a peroxide acceptor forming a complex which breaks down into acetic acid and carbon dioxide. Neil and Avery (1924) had previously shown that hydrogen peroxide accumulates in the media in which pneumococci are grown and that this accounts for the conversion of hemoglobin to methemoglobin. Kempner and Schlayer (1942) have shown that carbon dioxide increases the growth rate of pneumococci under certain conditions. Although there have been numerous reports on the growth requirements of these bacteria the effect of various compounds and conditions on their oxygen uptake has not been studied in detail. It is the purpose of this paper to present certain facts about the oxidation of various substrates by the pneumococcus.

EXPERIMENTAL

An avirulent Type I pneumococcus was grown for 18 hours in a medium containing meat extract 3.0 g., peptone 10.0 g., glucose 10 g. and NaCl 5.0 g. per liter. The glucose was added aseptically after autoclaving the rest of the medium. The bacteria were centrifuged, resuspended in 100 ml. of distilled water, centrifuged again, and finally resuspended in 0.05 M phosphate buffer at the desired pH so that approximately 0.5 ml. of packed bacteria were present in 10 ml. of buffer. Usually 0.5 ml. of this suspension was placed in each Warburg vessel which contained a final volume of 2.0 ml. The various substrates to be tested were dissolved in buffer and added from the side arms of the vessels. The washed bacteria without the addition of a suitable substrate had a very small oxygen uptake. This value is subtracted from the oxygen uptake figures obtained in the presence of certain substrates in the following experiments. The following compounds were tested. The natural and non-natural isomers of the amino acids, the lower and higher fatty acids, aliphatic and aromatic amines and aldehydes, succinic and fumaric acids, choline, inositol, peptone, meat extract, various proteins, lactic acid, pyruvic acid, glucose, fructose, mannose and ethyl alcohol. Only the last five substrates were oxidized. Addition of the known vitamins separately or together, or the addition of two or more substrates to the same vessel, did not have any effect on these results.

The oxidation of glucose, fructose and mannose

The amount of oxygen taken up by the bacteria in the presence of the sugars is dependent on the amount of sugar present and independent, within limits, of the number of bacteria. The end points of the oxidation are not sharply defined because each of the sugars produces a strong acid which lowers the pH and inhibits further oxygen uptake. This is consistent with the fact that the initial rate of oxidation is slower at pH 6.7 than at pH 7.8. Figure 1 shows, however, that fructose takes up about twice as much oxygen per mole as glucose or man-

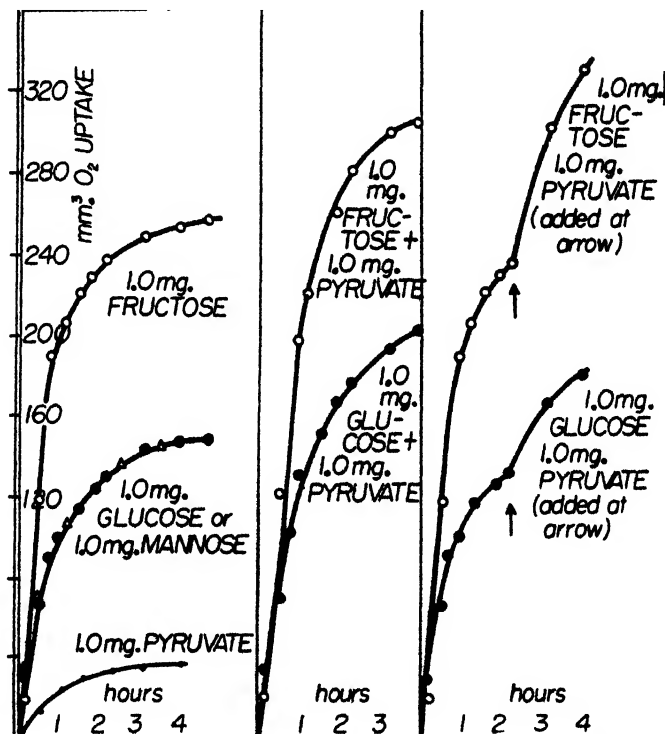


FIG. 1. THE OXIDATION OF FRUCTOSE, GLUCOSE, AND MANNOSE, AND THE EFFECT OF PYRUVIC ACID ADDED AT THE BEGINNING AND AT THE END OF THE OXIDATION. pH 7.8, 37°

nose. In confirmation of Sevag it was found that the addition of pyruvic acid to the bacteria oxidizing the sugars increases the oxygen uptake to a greater extent than can be accounted for by the oxidation of the pyruvic acid alone. The hydrogen peroxide formed when the sugars are oxidized is stable for some length of time because it is possible to add the pyruvic acid after the oxidation of the sugars is practically complete and still get an increased uptake. This is also shown in figure 1.

Since neither the amino acids nor the fatty acids are oxidized by this organism it was of interest to see whether the presence of these acids would affect the glucose oxidation, or whether they would be oxidized by the hydrogen peroxide.

The addition of amino acids is without effect, but the higher fatty acids markedly inhibit the oxidation of glucose. In a concentration of 1×10^{-4} M oleic acid which, because of its unsaturated bonds, might be expected to be oxidized by hydrogen peroxide actually inhibits the oxidation of glucose 58 per cent at pH 7.8 and 80 per cent at pH 6.7. This action of the fatty acids is similar to their action on *Blastomyces dermatitidis* (Bernheim, 1942) and is possibly accounted for by their surface activity which by adsorption onto the bacterial cell

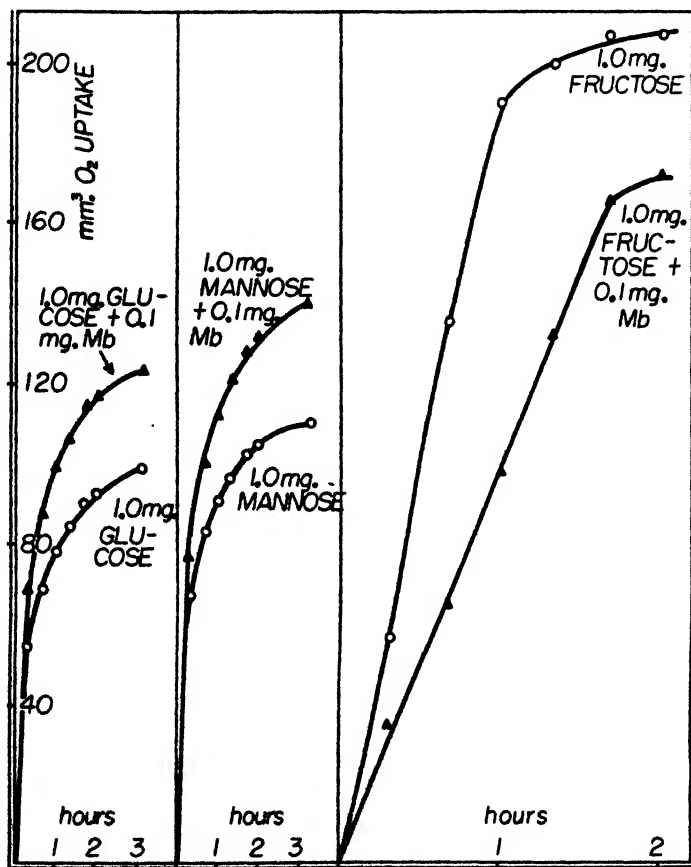


FIG. 2. THE EFFECT OF 1.3×10^{-4} M METHYLENE BLUE ON THE OXIDATION OF GLUCOSE, MANNOSE, AND FRUCTOSE. pH 7.8, 37°

prevents the access of glucose to its enzyme. The oxidation of pyruvic acid is also inhibited by oleic acid.

Methylene blue has a selective activity on the oxidation of the sugars. It slightly accelerates the oxidation of glucose and mannose but markedly inhibits that of fructose. This is shown in figure 2. The methylene blue was added in a concentration of 1.3×10^{-4} M and its action is specific because dyes of the indophenol and indigo sulfonate series are without effect. This fact indicates that fructose may be oxidized by an enzyme which is distinct from that which

oxidizes glucose and mannose. On the other hand monoiodoacetic acid in a concentration of 1.3×10^{-3} M inhibits the oxidation of all three sugars equally. The addition of lactic acid, peptone, ethyl alcohol or the complete medium has no effect on the oxidation of any of the sugars.

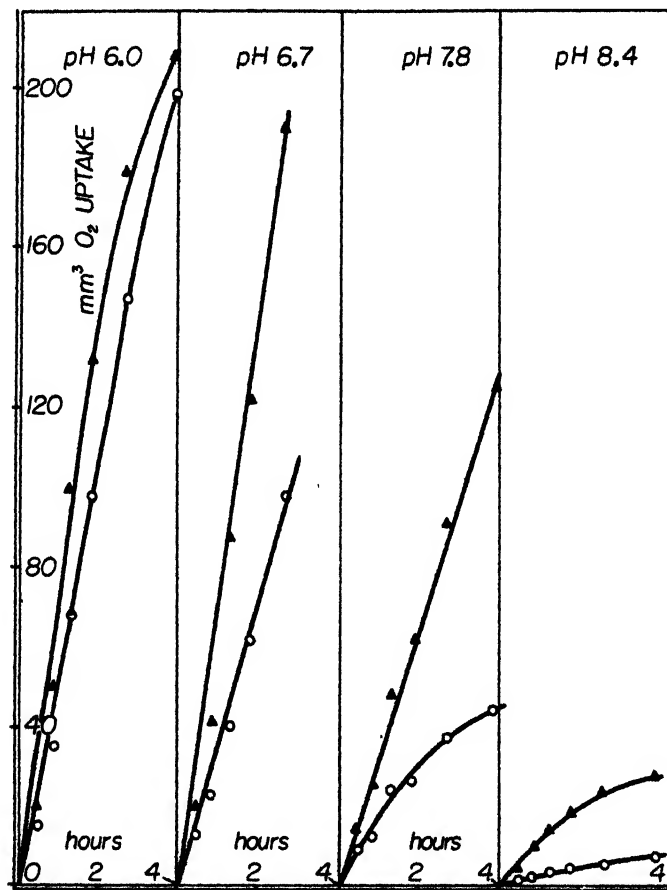


FIG. 3. THE EFFECT OF pH ON THE OXIDATION OF 4.0 MG. PYRUVIC ACID (CIRCLES) AND OF 4.0 MG PYRUVIC ACID IN THE PRESENCE OF 4.0 MG. l-LEUCINE (TRIANGLES)

The oxidation of pyruvic acid

At pH 7.8 the oxidation of pyruvic acid proceeds very slowly. As the pH is lowered the rate increases and it is greater at pH 6.0 than at pH 6.7. At all hydrogen ion concentrations the rate is much increased by the presence of amino acids, a tripeptide, peptone, meat extract and at pH 6.7 by certain proteins. For all the compounds except the proteins and certain amino acids the percentage increase is greater at pH 7.8 than in the acid range. This is shown in figure 3 for leucine. Because this effect on the oxidation of pyruvic acid has not been described before, it was studied in detail.

The amino acids are not all equally effective in increasing the rate of oxidation

of pyruvic acid. Leucine, isoleucine, phenylalanine and methionine are among the most active and are effective at pH 6.7 but show a larger percentage increase at pH 7.8. On the other hand tyrosine, glutamic acid, cystine and asparagin have more effect at pH 6.7. Glycine and valine have less activity and their action is less dependent on pH. Lysine is completely ineffective at any pH and alanine is almost completely ineffective. The non-natural isomers behave like their corresponding natural isomers. Table 1 shows these facts with certain selected amino acids. This group of compounds exhibits therefore some specificity of action although this cannot be correlated with their structure or iso-electric points.

TABLE 1

The effect of 4.0 mg. each of various amino acids on the oxidation of 4.0 mg. of pyruvic acid by pneumococci at pH 6.7 and 7.8 and 57°

TIME	PYRUVATE	+ l-LEUCINE	+ d-LEUCINE	+ l-ISO-LEUCINE	+ d-ISO-LEUCINE	+ l-VALINE	+ d-VALINE	+ l-ALANINE	+ d-ALANINE	+ l-PHENYLALANINE	+ d-PHENYLALANINE	+ d-LYSINE	+ dl-METHIONINE	+ l-HISTIDINE	+ l-TYROSINE	+ l-ASPARAGIN	+ l-GLUTAMIC ACID	+ dl-SERINE
pH 7.8																		
min.	mm ³	mm ³	mm ³	mm ³	mm ³	mm ³	mm ³	mm ³	mm ³	mm ³	mm ³	mm ³	mm ³	mm ³	mm ³	mm ³	mm ³	mm ³
35	21	27	38	49	28	30	28	21	22	29	31	22	31	21	30	22	21	30
80	35	59	85	80	57	65	46	37	34	61	63	35	68	35	49	39	35	72
140	57	117	134	132	116	110	80	59	60	120	122	59	127	60	65	71	58	120
190	73	161	182	182	157	140	105	76	71	170	175	74	180	80	82	82	75	152
270	98	218	230	237	223	170	146	102	100	228	230	96	237	112	112	120	101	181
pH 6.7																		
30	20	27			25		25	21	20	32		20	21	24	35	28	29	
75	44	72			64		50	50	46	78		45	58	52	72	52	76	
110	85	141			118		122	95	87	135		84	116	111	110	101	147	
180	124	195			172		177	135	125	182		125	173	160	171	158	208	
230	158	231			220		223	170	160	230		160	224	209	210	201	254	

In an attempt to find the explanation for this effect, the oxidation of pyruvic acid was followed by measuring the oxygen uptake and carbon dioxide production, and by estimating the amount of the acid remaining at the end of the experiment, by the method of Clift and Cook (1932). In a typical experiment carried out at pH 6.7 in the presence of 3.16 mg. of pyruvic acid the following values were obtained after the oxidation had been allowed to proceed for five hours. From the O₂ uptake figures 1.00 mg. of pyruvic acid was oxidized; from the CO₂ production figures 1.09 mg. were decarboxylated; and from the bisulfite estimation 1.14 mg. had disappeared. The corresponding values in the presence of 4.0 mg. of l-leucine were 1.72, 1.81, and 1.72 mg. In the presence of 4.0 mg. l-glutamic acid the corresponding values were 1.94, 1.96, and 1.82 mg. This

experiment indicates that the amino acid accelerates the oxidative decarboxylation of pyruvic acid.

The effect of the concentration of amino acid was next determined. Increasing the amount of amino acid from 0.1 to 2.0 mg. in 2.0 ml. increases the percentage acceleration but not proportionally. A further increase in concentration is without effect. This indicates that the amino acid is not participating as a substrate in the reaction. To obtain further information about this point the bacteria were precipitated at the end of the oxidation with 10 per cent trichloroacetic acid and centrifuged. An aliquot of the liquid was taken and the amino nitrogen determined in the van Slyke apparatus. In this experiment at pH 7.8, 2.0 mg. of pyruvic acid took up 37 mm³ of oxygen in five hours. In the presence of 2.0 mg. of l-leucine 101 mm³ were taken up. The amino nitrogen added at the beginning as leucine was 0.214 mg. and at the end of the experiment 0.205 mg. was recovered. These figures indicate that the leucine is not deaminated and are consistent with the hypothesis that it acts as a catalyst and increases the oxidation of pyruvic acid.

In order to determine whether a free amino group was necessary to obtain the accelerating effect on the pyruvic acid oxidation a number of N-substituted amino acids were tested. In the following experiment at pH 7.8 the pyruvic acid had taken up 60 mm³ of oxygen in five hours in the presence of the bacteria alone. When 4.0 mg. dl-valine were added, 95 mm³ were taken up, and when 4.0 mg. dl-N-methylvaline were added only 58 mm³ were taken up. The corresponding values for 4.0 mg. each of certain other amino acids are as follows: dl-leucine, 168 mm³; dl-N-methylleucine, 97 mm³; glycine, 103 mm³; N-methylglycine, 84 mm³; N-ethylglycine, 82 mm³; dl-phenylalanine, 149 mm³; and dl-N-acetyl-phenylalanine 55 mm³. It is therefore apparent that substitution on the nitrogen atom lowers or completely prevents the acceleration. A comparison between the action of tyrosine and tyramine showed that the latter was completely inactive which indicates that the carboxyl group is also necessary for activity.

The effect of the number of bacteria on the amino acid acceleration is shown in the following experiment done at pH 6.7. At the end of five hours in the presence of 0.2 ml. of a suspension of pneumococci the pyruvic acid had taken up 68 mm³ of oxygen. The addition of 3.0 mg. l-leucine caused an uptake of 113 mm³, an increase of 66 per cent. In the presence of 0.4 ml. of the suspension the corresponding values were 156 mm³ and 208 mm³, an increase of 33 per cent. Thus, the greater the number of bacteria the less the percentage increase which might be explained on the assumption that the bacteria contain a substance which may act like the amino acid. The oxidation of ethyl alcohol is not affected by the presence of the amino acids. Since the first step in the oxidation of alcohol is acetaldehyde, which is also probably formed in the decarboxylation of pyruvic acid, this fact indicates that the accelerating effect of the amino acids on pyruvic acid is concerned with the primary decarboxylation and not with the subsequent oxidation of acetaldehyde to acetic acid. Neither thiamine nor a mixture of vitamins containing thiamine, riboflavin, pantothenate, pyridoxine and choline affects the oxidation of pyruvic acid or the amino acid acceleration.

The effect of proteins

Table 2 shows the effect of various proteins on the oxidation of pyruvic acid. Crystalline serum albumin and hemoglobin prepared by laking thoroughly washed human red cells cause a marked acceleration. On the other hand crystalline egg albumin is inactive and casein actually causes an inhibition. This latter protein has no effect on the oxidation of glucose. The serum albumin effect can only be demonstrated at pH 6.7 and the hemoglobin effect is much greater at this pH than at 7.8. On a molar basis the proteins are more effective than the amino acids. Heating the serum albumin at 100° for varying lengths of time causes it to lose its accelerating effect and its presence then may cause some inhibition of the pyruvic acid oxidation. This is also shown in table 2. The proteins therefore show a marked specificity. In all other respects their action is analogous to that of the amino acids. Peptone and a tripeptide, leucylglycylglycine, also accelerate the pyruvic acid oxidation and on a molar

TABLE 2

The effect of 4.0 mg. each of various proteins on the oxidation of 4.0 mg. of pyruvic acid by the pneumococci at pH 6.7 and 37°

The serum albumin was heated at 100° in buffer pH 6.7

TIME	PYRUVATE	+ SERUM ALBUMIN	+ SERUM ALBUMIN HEATED 5 MIN.	+ SERUM ALBUMIN HEATED 10 MIN.	+ SERUM ALBUMIN HEATED 15 MIN.	+ HEMO- GLOBIN	+ EGG ALBUMIN	+ CASEIN
min.	mm ²	mm ²	mm ²	mm ²	mm ²	mm ²	mm ²	mm ²
45	32	51	26	26	24	53	32	10
75	65	96	55	53	47	102	63	25
120	115	147	101	93	81	153	109	49
180	179	206	163	151	131	210	173	72

basis their activity is between that of the proteins and amino acids. They resemble certain amino acids in that they are active in both acid and alkaline solutions, but like serum albumin, peptone loses its activity when heated. It requires an hour in the autoclave to inactivate peptone regardless of the pH.

DISCUSSION

These results with pneumococci can be compared with those obtained with *Blastomyces dermatiditis* (Bernheim, 1942). Both organisms are unable to oxidize fatty and amino acids. In both cases fatty acids inhibit the oxidation of other substrates. Amino acids increase the control oxygen uptake of *Blastomyces*, which is relatively large, without being oxidized themselves. They have no effect on the control oxygen uptake of the pneumococci, which is very small, but accelerate the oxidation of pyruvic acid. The demonstration of these facts indicates a means by which substances, not themselves oxidized, may indirectly affect the oxidative metabolism of the cell. The exact mechanism by which amino acids and certain proteins accelerate the pyruvic acid oxidation in pneumococci has not been determined. It seems possible that they might act

as carbon dioxide receptors forming carbamino compounds. All attempts however to prove the presence of such compounds have failed.¹ The possibility also exists that the amino acid or protein in some way facilitates the access of pyruvate to its enzyme perhaps by affecting the permeability of the cell. It was shown that they had no effect on the pH which was held constant throughout the experiment by the buffer present.

SUMMARY

1. The conditions which affect the oxidation of glucose, fructose and mannose by a Type I avirulent pneumococcus have been described. The higher fatty acids inhibit the oxidation of all three sugars. Methylene blue inhibits the oxidation of fructose but slightly accelerates that of glucose and mannose. All the sugars are oxidized more slowly at pH 6.7 than at pH 7.8.

2. Pyruvic acid is oxidized more rapidly at pH 6.7 than at pH 7.8. Addition of certain amino acids which are not themselves oxidized or of certain proteins, peptones or peptides markedly increases the oxidation of pyruvic acid.

3. The conditions in which this accelerating effect occur are described in detail.

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¹ At the end of the experiment in which pyruvic acid was oxidized in the presence of an amino acid the mixture was brought to pH 11-12 and excess BaCl₂ added to remove free carbon dioxide. The carbamino compound if present should not be precipitated but should yield carbon dioxide on acidification after the removal of the barium carbonate and bacteria by centrifugation. This did not occur.

TWO AGAR-LESS MEDIA FOR THE RAPID ISOLATION OF CORYNEBACTERIUM AND NEISSERIA

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This study was initiated to find agarless media suitable for meningitis and diphtheria surveys in the field, because of the large quantity of agar needed for this work while agar stocks-on-hand are rapidly diminishing. These media had to be simple to make, easy to use, and permit isolation and study of *Corynebacterium diphtheriae* and *Neisseria intracellularis* with as great speed and accuracy as agar-containing media. Media fulfilling these requirements have been developed.

Media made of various gums, such as gum Karaya, were tried with unsatisfactory results as solutions of such gums were liquid at 37°C. The easy availability of animal blood serum turned the investigation in that direction. A very simple, quick and effective technic for making inspissated serum plates, as used in this laboratory, is described below in Section C.

SECTION A. SERUM-CASAMINO ACIDS MEDIUM FOR *N. INTRACELLULARIS*

Loeffler medium failed entirely in the initial isolation and detection of *N. intracellularis* and supported old laboratory strains with difficulty.

Blood cells, hemoglobin solutions, and vitamin enrichments were tried in serum formulae with unsatisfactory results.

The following formulae incorporating casamino acids promoted excellent growth. The formulae are given in order of growth efficiency.

I

Casamino acids, Difco.....	1.5 g.
Proteose # 3, Difco.....	1.5 g.
Glucose.....	1.0 g.
Sodium phosphate, dibasic U.S.P.....	1.0 g.
Distilled water.....	25 ml.
Glycerol.....	2 ml.

Bring into solution with gentle heating, cool, and add

Hog serum.....	75 ml.
----------------	--------

Titrate to pH 7.6 with NaOH N/1

II

Casamino acids, Difco.....	2.0 g.
Beef Extract.....	0.3 g.
Glucose.....	1.0 g.
Sodium phosphate, dibasic U.S.P.....	1.0 g.
Distilled water.....	25 ml.
Glycerol.....	2 ml.

Bring into solution by gentle heating, cool, and add

Hog serum.....	75 ml.
----------------	--------

Titrate to pH 7.6 with NaOH N/1

This laboratory adds 0.1 ml. Bromthymol Blue 1% adjusted with sodium hydroxide according to Clark (1920). The indicator gives a more satisfactory background for the watery *Neisseria* colonies and demonstrates glucose utilization. Direct titration with sodium hydroxide of the whole medium, to which indicator has been added, obviates the use of hydrogen ion testing equipment in the field.

The blood serum used is that collected at a local slaughter house. Chloroform 1:50 is added as a preservative. Hog serum is used exclusively as it gives a firmer coagulum at the more alkaline levels preferred, pH 7.4–pH 7.8. This has been well brought out by the work of Medalia and others (1931) and by Laybourn (1935).

The casamino acids used are those described by Mueller and Johnson (1941) and Mueller and Hinton (1941) and are manufactured by the Difco Laboratories, Detroit.

Thirteen to 15 ml. of the medium are poured into clean petri dishes which need not be previously sterilized. This medium is inspissated and sterilized in one operation in the autoclave (Section C). The contaminants usually introduced into pour plates are absent even though the medium is made under field conditions as the sterilized medium is not exposed to the air until it is to be seeded with material for investigation.

Plates are incubated at 37° under CO₂ generated by burning a candle in an airtight can. Colonies of *N. intracellularis* from spinal fluid are visible early and average 2–3 mm. in diameter at 24 hours, permitting a confirmatory agglutination test at that time. Colonies are typical, watery, convex, entirely edged, easily emulsifiable and give positive oxidase reactions. Confluence of colonies is to be expected in heavily seeded plates.

SECTION B. SERUM-TELLURITE MEDIUM FOR CORYNEBACTERIUM

The advantages of potassium tellurite, incorporated in culture medium, to facilitate diphtheria identification has been described by many investigators (Gilbert and Humphreys, 1926; Cooper *et al.*, 1940). The medium described here required no agar, may be made easily in the field, and has been found to be as efficient as other tellurite media for diphtheria work.

Proteose #3, Difco.....	0.5 g.
Glucose.....	0.2 g.
Distilled water.....	25 ml.
Glycerol.....	2 ml.

Bring into solution with gentle heating, cool, and add

Hog serum.....	75 ml.
Potassium tellurite 1% solution.....	2.5 ml.

Titrate to pH 6.4–6.6 with HCl N/1.

Pour 13–15 ml. of the medium into clean petri dishes. Inspissate and sterilize according to directions in Section C. After sterilization the pH of the medium will approximate 7.0–7.2.

Corynebacterium colonies usually make their appearance in 24 hours. They are low convex, glistening colonies of about 1 mm. diameter, light to dark gray in color against the cream-colored medium. The plate may be seeded directly with the cotton swab used to take material from nose or throat, since the growth of nose and throat bacteria, other than that of *Corynebacterium*, is markedly inhibited.

SECTION C. INSPISSATION AND STERILIZATION OF SERUM MEDIUM PLATES

The single chambered bacteriological autoclave

1. Preheat the autoclave by running in steam for 5 minutes at 15 pounds pressure and allow the pressure to return to zero.
2. Line the floor of the autoclave or the shelves of a rack with several layers of dry newspaper and place plates in the autoclave in a single layer. If hardware cloth is used instead of solid metal shelves, paper lining is unnecessary. Plates should never be placed directly onto a good heat conductor nor piled on one another.
3. Close ALL water, steam and air outlets.
4. Allow steam to enter slowly so that 15 pounds pressure is reached in 3 minutes. Keep original air entrapped.
5. The plates are allowed to remain for an additional 7 minutes in the closed system. The valve controlling the water exit is then opened slightly to replace entrapped air with steam and to allow the condensed water and a small amount of steam to escape gradually so that the pressure is not appreciably changed. The serum-tellurite medium is sterilized 10 minutes more: a total of 20 minutes. The serum casamino acids medium is sterilized 15 minutes more: a total of 25 minutes.
6. At the end of sterilization, close the steam inlet and open ALL outlets quickly, the largest first, opening the safety valve if necessary to reduce pressure to zero in 5-10 seconds. Remove the plates as soon as possible from the autoclave.

Pressure cooker type field autoclave

1. Pour a small amount of water into the bottom of the cooker and allow it to come to a boil.
2. Place the plates of medium in a single layer on the bottom or in single layers in a rack in the autoclave. Do not pile plates on one another.
3. The lid is clamped on quickly, the petcocks closed and the pressure brought slowly to 15 pounds.
4. The plates are allowed to remain 10 minutes from the beginning of pressure rise in the closed system. The air vent is then opened slightly so that there is no appreciable change in pressure. The serum-tellurite medium is sterilized 10 minutes more: a total of 20 minutes. The serum-casamino acids medium is sterilized 15 minutes more: a total of 25 minutes.
5. At the end of sterilization, open the outlets in the lid and start opening the cover clamps as quickly as possible. Remove the plates immediately from the autoclave.

Double chambered dressing type autoclave

This type of autoclave is not recommended unless the water trap line is equipped with a manually operated valve in lieu of or in addition to the thermostatic valve. It is then operated similarly to the single chambered bacteriological autoclave.

1. Close ALL outlets, including the valve between the inner and outer chambers.
2. Place the plates of medium on the shelves of the rack or on the bottom of the autoclave which has been padded with several layers of dry newspaper.
3. Run steam into the outer jacket until 20 pounds pressure is reached.
4. Open the valve between the inner and outer chambers slowly and allow an inner pressure of 15 pounds to be reached in 3 minutes.
5. The plates are allowed to remain an additional 7 minutes in the closed system. The valve on the water outlet trap is then opened slightly so that there is no appreciable change in pressure. The serum-tellurite medium is allowed to sterilize 10 minutes more: a total of 20 minutes. The serum-casamino acids medium is allowed to sterilize 15 minutes more: a total of 25 minutes.
6. At the end of sterilization, close the steam inlet valve and open ALL outlets quickly, opening the largest first. Open also the safety valve, if necessary, to reduce pressure to zero in 5-10 seconds. Remove plates immediately.

Experience in developing these media has shown that unless the directions laid down are followed exactly, these poor results may be encountered:

Bubbles in the finished medium which may be due to:

1. The plate resting on a heat conductor or a *wet* paper.
2. Failure to entrap air during inspissation period, e.g. when outlet valve is not securely closed.
3. An acidity greater than that recommended.
4. Too rapid rise in the steam pressure so that inspissation begins before the chloroform in the serum has boiled off.

Fragmentation after inspissation which may occur if the pressure at the end of sterilization is reduced *too slowly*.

Plates of too soft consistency may be due to:

1. An alkalinity higher than that recommended.
2. Insufficient heating of the medium because of poor circulation or low pressure of steam.

Blackening of serum-tellurite medium which may be due to:

1. Higher alkalinity than that recommended.
2. Plate in contact with heat conductor or wet paper. When the serum-tellurite plate is too *acid* the tellurium will not be reduced by bacterial growth.

SUMMARY

Two agarless media for the detection of *Corynebacterium diphtheriae* and *Neisseria intracellularis* are described and are recommended for survey work in the field as well as for diagnosis in the clinical laboratory. The media have these advantages:

1. They promote excellent growth in a short time, curtailing the time spent on a survey.
2. They are easy to make.
3. They do not require the use of sterile glassware nor reagents.
4. The usual pour plate contaminants are absent.
5. They are composed of reagents that are available.

In concluding, I wish to express my appreciation to Lt-Comdr. Robert W. Babione (MC) USN for his helpful criticism in reading this paper and through whose encouragement and advice this work was made possible, and to Lieut. James N. DeLamater (MC) USN, and to other members of the West Coast Epidemiological and Mobile Unit for their helpful assistance rendered me in this work.

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EXPERIMENTAL VARIATION OF NICOTINAMIDE REQUIREMENT OF DYSENTERY BACILLI¹

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With increasing knowledge of vitamin requirements and of vitamin synthesis by various groups of microorganisms the question of variation with respect to such factors naturally arises. On the basis of findings thus far it seems reasonably clear that such variation occurs under natural conditions. In every instance in which a number of representative strains of the same group or of the same species has been studied, some difference has been found in the requirement between strains as they first come under laboratory cultivation. While the majority consistently show a certain definite requirement, a few differ and either are less exacting or exhibit added requirements. It is usually assumed that these differences have arisen at some time in the past history of the microorganism. At present we have little information as to the conditions or time required to effect such changes.

With respect to metabolites other than the vitamins, such as amino acids and sugars, it is now well recognized that the nutritional requirement and the enzyme equipment of the bacterial cell can be changed at times by alteration of the substrate.

Concerning the vitamins, evidence based on experimental work is quite limited but there are several reports which show that changes of similar nature can be brought about by altering the conditions of laboratory cultivation. Wood, Anderson and Werkman (1938) found that several transfers in a basal medium deficient in thiamin were sufficient to "train" a strain of *Propionibacterium pentosaceum* to dispense with thiamin. The "trained" culture synthesized appreciable quantities of the vitamin, as determined by microbiological methods (Silverman and Werkman, 1939).

Leonian and Lilly (1942) succeeded in inducing ten strains of *Saccharomyces cerevisiae* to grow readily without several factors which were originally needed for prompt growth. Thiamin, pyridoxine, inositol and pantothenic acid could be dispensed with, so that in most cases only biotin was needed and even biotin could be omitted in one instance. Evidently this adaptation was brought about quite readily, four to seven passages usually sufficing to obtain growth in the absence of a given vitamin. Evidence was advanced to show that after such "training" the yeasts synthesized the omitted factors.

An interesting example of the opposite sort, representing a loss rather than a gain of synthetic ability has been reported (Beadle and Tatum, 1941; Tatum and Beadle, 1942). By x-ray treatment, mutant strains of *Neurospora* were obtained

¹ This investigation was aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

which were characterized by loss of the property to synthesize either pyridoxine, the thiazole constituent of thiamin, or para-aminobenzoic acid.

In the present investigation we have studied variation in nicotinamide requirement of dysentery bacilli, particularly with respect to the derivation of variants able to dispense with nicotinamide.

CULTURES

Four strains of *Shigella paradysenteriae* were used: Flexner 76, Strong 3, Sonne 8 and Sonne 269. These strains had been used in previous work in this laboratory and in numerous tests over the past several years always required nicotinamide (Koser, Dorfman and Saunders, 1938; Dorfman *et al.*, 1939). The last three strains grow readily in an ammonium-salt-glucose medium with the added accessory factor. Strain 76 requires certain amino acids. Stock cultures of these organisms were always kept on agar slants and transferred about once a month with refrigeration in the interim.

MEDIA

For the experiments on variation two basal media of known chemical composition were used. One supplied a variety of amino acids, the other was limited to inorganic nitrogen.

The amino acid medium was composed of 19 amino acids, 0.2 per cent dipotassium hydrogen phosphate, 0.01 per cent magnesium sulphate, 0.8 per cent sodium chloride and 0.3 per cent glucose. The individual amino acids were supplied for the most part in the same amount as in our earlier work, except serine and threonine which were added in the amount of 15 milligrams per liter. This medium was adjusted to pH 6.8 to 7.0, tubed in 5 ml. amounts and sterilized in the autoclave.

The simpler synthetic medium was composed of 0.2 per cent diammonium hydrogen phosphate, 0.15 per cent potassium dihydrogen phosphate, sodium chloride, magnesium sulphate and glucose as before. The pH was 6.8 to 7.0. It was tubed and sterilized as the preceding medium.

Nicotinamide was sterilized by filtration through glass filters and when needed for certain of the experiments was added to the previously autoclaved basal medium.

METHODS

Two methods were used in attempts to develop cultures capable of growth without added nicotinamide. These are similar in principle to those used by other investigators in previous studies of variation in nutritional requirements. They are summarized below.

I. This consisted of heavy inoculation of a tube of synthetic medium without nicotinamide, followed by attempted serial transfer in the same medium. Serial transfers were started with varying-sized inocula (a) immediately and (b) after intervals of incubation at 37°C. of from 7 to 28 days.

II. This method consisted of serial transfers in the presence of decreasing sub-optimum amounts of nicotinamide, followed by attempted transfers without nicotinamide.

Both the amino acid medium and the ammonium phosphate medium were used in connection with each of these methods.

When mass or bulk inoculation (method I) was used, a tube of synthetic medium was inoculated heavily from a 24-hour agar slant culture. This large inoculum produced dense turbidity in the tube and of course resulted in the introduction of an indefinite quantity of nicotinamide and other factors along with the cells.

The use of an initial large inoculum followed by subculture from this tube of graded amounts to start separate series of transfers was made with the idea that cells capable of growing without nicotinamide, or those potentially able to develop this capacity, might be present in very small numbers in relation to the total. Thus, large inocula would be needed to detect such cells while the additional use of smaller inocula of varying size might serve to give a rough idea of their approximate numbers.

Continued incubation of the bulk inoculation tube at 37°C. followed by later attempts at serial cultivation seemed desirable because of the possibility of alteration in metabolic capacities of the cells during the holding period. In this case also, it seemed best to transfer graded inocula when attempting to detect by serial cultivation cells capable of growing without added nicotinamide. The number of cells carried over in each instance was determined by plate counts using glucose veal-infusion agar.

Since with the larger numbers of cells a considerable quantity of nicotinamide was inevitably carried over when starting a series of transplants, growth in the serial transfers without nicotinamide was not considered significant unless it appeared regularly in later transfers. Successive transfers were made by carrying over 0.1 ml. from a 5.0 ml. culture tube. The dilution of one-fiftieth with each transfer soon resulted in elimination of nicotinamide carried over with the original inoculation. Earlier work with the dysentery bacilli showed that 0.0005 to 0.001 μ g nicotinamide per ml. of liquid medium is needed to support a barely perceptible growth.

Distinct growth in the serial transfers beyond the fourth or fifth transplant was regarded as significant, though in the final tabulation of results growth without added nicotinamide was not recorded as definitely positive unless the culture could be transferred continuously through 20 subcultures.

EXPERIMENTAL RESULTS

Table 1 summarizes the attempts to induce growth in the amino acid medium following mass inoculation of the first tube. It is evident that continuous growth without the addition of nicotinamide was obtained in some cases. This was true of all four cultures. In certain instances serial transfer was accomplished in a relatively large proportion of cases; six, seven, eight or even nine instances in a total of twelve attempts. With a lengthening of the incubation period, growth could be established in subsequent successive transfers by the use of much smaller numbers of cells than when the transfers were started immediately.

Taking Sonne strain 8 as an example, when the serial transfers were started immediately (0 days at 37°C.) the carrying over of approximately 50 million cells

resulted in establishing 7 separate series, out of 12 attempts, in which continuous growth occurred through 20 successive transfers without added nicotinamide. When half-a-million cells were used for the initial inoculum continuous growth

TABLE 1

Summary of attempts to secure growth in amino acid medium without nicotinamide, following mass inoculation

ORGANISM USED	DAYS AT 37° BEFORE SERIAL TRANSFERS STARTED	NUMBER OF CELLS IN INOCULUM TO START SERIAL TRANSFERS	NUMBER OF SERIES ATTEMPTED	NUMBER OF SUCCESSFUL SERIES*
Sonne 8. Initial inoculation 510 million cells per ml.	0	51,000,000	12	7
	0	510,000	12	0
	7	700,000	12	9
	7	70,000	12	0
	7	7,000	12	0
	28	2,000	4	2
	28	200	12	0
	28	20	12	0
Sonne 269. Initial inoculation 594 million cells per ml.	0	59,400,000	12	5
	0	594,000	12	0
	7	2,500,000	12	9
	7	250,000	12	0
	7	25,000	12	0
	28	10,500	4	2
	28	1,000	12	0
	28	100	12	0
Strong 3. Initial inoculation 492 million cells per ml.	0	49,200,000	12	6
	0	490,000	12	0
	7	880,000	12	8
	7	88,000	12	0
	7	8,800	12	0
	28	630	4	3
	28	63	12	0
	28	6	12	0
Flexner 76. Initial inoculation 467 million cells per ml.	0	46,700,000	12	2
	0	467,000	12	0
	7	1,100,000	12	1
	7	110,000	12	0
	7	11,000	12	0
	28	340	12	0
	28	34	12	0

* Series were designated as successful only after growth was obtained in 20 successive transfers without added nicotinamide.

could not be induced. Apparently only a very small percentage of cells can adjust themselves to growth without added nicotinamide.

After the original mass inoculation tube had been held at 37°C. for 7 days the number of viable cells, as determined by plate counts, had fallen from 51,000,000

to 700,000 in 0.1 ml. This is approximately the number which had previously given consistently negative results, yet when this number of cells was now used to start each of 12 different series, variants appeared in 9 of them. After 28 days at 37°C. agar plate counts revealed the presence of but 2,000 viable cells in 0.1 ml. of the original mass inoculation tube. Two instances of successful growth without added nicotinamide were obtained in 4 attempts when these cells were used to start the series, but not when smaller numbers were employed.

Thus, with continued incubation of the original tube the number of viable cells decreased, but there appeared to be an increase in the proportion of cells capable of growing, or of developing the capacity to grow, without added nicotinamide. We have no definite evidence whether this was due to a gradual alteration in metabolic capacity of surviving cells or whether it was due merely to slow multiplication of a few cells which originally possessed the additional capacity.

TABLE 2

Summary of attempts to secure growth in ammonium phosphate medium without nicotinamide, following mass inoculation

ORGANISM USED	DAYS AT 37° BEFORE SERIAL TRANSFERS STARTED	NUMBER OF CELLS IN INOCULUM TO START SERIAL TRANSFERS	NUMBER OF SERIES ATTEMPTED	NUMBER OF SUCCESSFUL SERIES
Sonne 8	0	54,000,000	5	0
	7	2,500,000	5	0
	28	1,600,000	3	0
Sonne 269	0	50,000,000	5	0
	7	9,300,000	5	0
	28	4,500,000	3	0
Strong 3	0	44,000,000	5	0
	7	5,200,000	5	0
	28	2,600,000	3	0

The tubes receiving the mass inoculation contained 540 million, 500 million and 440 million cells per ml. respectively at the start of the experiment.

Quite different results were secured when the same procedure was applied to the simpler synthetic medium in which ammonium phosphate replaced the amino acids. From previous work it was known that 3 of the 4 cultures grew readily in the ammonium phosphate medium when small amounts of nicotinamide were supplied. The fourth culture, Flexner 76, was not included because it needed certain amino acids for growth in addition to nicotinamide. Results for the three cultures are given in table 2.

Attempts to induce growth in the simpler medium in the absence of nicotinamide failed. This evidently could not be attributed to the use of an insufficient number of cells. The numbers used to start the successive transfers immediately (0 days at 37°C.) are quite similar to those used in the case of the amino acid medium, while larger numbers survived to 7 and 28 days than in the preceding experiment.

This striking difference in results seems of considerable theoretical interest. In the presence of amino acids certain cells apparently either synthesize nicotinamide or get along without it, perhaps utilizing the amino acids in some way to satisfy a metabolic function performed by nicotinamide. When the ammonium salt was substituted for amino acids the synthesis or the substitution could not be accomplished. It follows that in attempting to induce variants or to "train" cultures to multiply without an added accessory factor, the composition of the basal medium used is important and may determine the results obtained.

Serial transfers

The amino acid synthetic medium was used for the first experiments involving this procedure (method II). Tubes were inoculated with cells taken from 24-hour agar cultures. The inoculum was so adjusted that from 50,000 to 100,000 cells per ml. of medium were added to the first tube of the series. Our previous experience with these organisms had shown that following an inoculum of this size, growth does not appear in the synthetic medium without added nicotinamide and this was confirmed by control tests during the present work.

To the first several tubes of each series 0.002 μ g nicotinamide per ml. of medium was added. This amount is distinctly less than that necessary for optimum growth but permits the attainment of light turbidity, usually within 24 hours. After development of visible growth transfers were made to the next tube in the series, using 0.1 ml. of the culture. During the first fifteen to twenty transfers the amount of added nicotinamide was gradually decreased to 0.0005 μ g per ml. If light growth still persisted, the accessory factor was then omitted entirely. If growth occurred after its omission, an attempt was then made to carry the culture through 15 to 20 additional transfers in the amino acid medium without added nicotinamide.

From each of the four dysentery cultures variant strains were obtained capable of such serial transfer. These variants were obtained in each of duplicate series of transfers from cultures 3, 8 and 269, and were obtained in one of two series from culture 76.

The ammonium phosphate basal medium was also used for similar experiments. Suboptimum amounts of nicotinamide were added to the tubes, the amount was decreased after several transfers and the compound finally omitted as in the preceding experiments. In this medium most of the attempts at serial passage failed when nicotinamide was omitted. In a few instances light growth was obtained through twenty transplants without nicotinamide but it was so light as to leave the conclusions somewhat in doubt.

In general the results of the serial transfers in the presence of decreasing concentrations of the accessory factor were similar to the mass inoculation experiments. It was found to be much more difficult or impossible to establish serial growth without nicotinamide in the simple ammonium phosphate medium than in the medium supplying a variety of amino acids.

Comparative growth

The rate and luxuriance of growth of the variant strains was compared with that of the original parent cultures at intervals. This was done usually at every

fifth or tenth transfer during serial cultivation of the variants in the amino acid medium. At these times parallel inoculation of the original culture was made into the amino acid medium and also both the variant and the original were introduced into the same medium with 0.1 μg per ml. of nicotinamide. Thus, the growth of variant and original, both with and without nicotinamide, could be compared.

TABLE 3
Comparative growth of original and "trained" strains in amino acid medium, with and without added nicotinamide

ORGANISM USED	AMINO ACID MEDIUM WITHOUT NICOTINAMIDE		AMINO ACID MEDIUM PLUS NICOTINAMIDE 0.1 μg PER ML
	24 hours	48 hours	24 hours
Sonne 8			
10th transfer.....	++	++	++++
50th transfer.....	++	++	+++
original culture.....	0	0	++++
Sonne 269			
10th transfer.....	++	++	++++
50th transfer.....	++	++	++++
original culture.....	0	0	++++
Strong 3			
15th transfer.....	++	+++	++++
50th transfer.....	++	++	++++
original culture.....	0	0	++++
Flexner 76			
15th transfer.....	++	++	++++
50th transfer.....	++	++	+++
original culture.....	0	0	++++

0 = no visible growth, + to ++++ light to pronounced turbidity.

In tubes with ++ growth, the number of cells usually was between 30 and 45 million per ml. as determined by agar plate counts; with ++++ growth it was found to be 100 million or greater per ml.

Continued incubation of the cultures beyond 48 hours produced no appreciable increase in turbidity.

Representative results for all four strains are summarized in table 3. This gives the growth of the variant at either the tenth or fifteenth transfer without nicotinamide and also at the fiftieth such transfer, when the series was usually terminated. Results for the original stock cultures are also shown. These cultures failed to grow in every test and so only one result is given for them, though they were tested along with the variants at every fifth or tenth transfer. During the serial cultivation of the variants the original cultures had been maintained on nutrient agar slants, transferring about once a month with storage in the ice box between transfers.

In all cases the variants, once established by serial transfer, grew in the amino

acid medium without nicotinamide while the original cultures failed to grow. A rather striking feature of these comparative tests, and one which was encountered regularly, was that the "trained" variants never attained as luxuriant growth as in the presence of nicotinamide. The lighter growth in the amino acid medium appeared promptly, usually within 24 hours, and was not appreciably enhanced by continued incubation of the tubes beyond 48 hours. In spite of this, however, these cultures could be maintained through fifty successive transfers. Also, the lighter growth of the variant strains was not appreciably increased with continued transfer, but remained approximately at the same level from the early transfers to the end of the series. Readings of growth by visual inspection, as given in table 3, were checked in confirmatory tests by readings in an Evelyn colorimeter with similar results.

The variants used in the tests shown in table 3 were obtained from the mass inoculation experiments. Similar results, not shown in tabular form, were obtained also with the other variants secured through serial transfers in the amino acid medium in the presence of decreasing amounts of nicotinamide.

In general it may be said of the variants that the ability to grow in the amino acid medium became evident within the first few transfers, that growth was not materially enhanced by subsequent passages through fifty transfers and that it never attained the luxuriance shown by cultures in the presence of optimum amounts of nicotinamide.

These results possess some interest in relation to the question of synthesis of nicotinamide. It is usually assumed that a microorganism when growing without a particular accessory factor is able to synthesize the compound. There is now considerable evidence that this is true in many cases. Our observations on the limited growth of the variants led us to suspect that these strains might not be able to synthesize nicotinamide. Rather they might be making use of certain amino acids in some way to satisfy partially the functions performed by nicotinamide and thus be able to grow to a limited extent. It seemed reasonable to believe that had they acquired the ability to synthesize small amounts of nicotinamide, sufficient for moderate growth, then the synthesis would proceed further, or those cells able to synthesize the compound would outgrow the others in subsequent transfers, so that eventually sufficient amounts would be formed to permit of the usual luxuriant growth. Such, however, was not the case through fifty successive transfers.

These considerations led us to determine whether nicotinamide or diphosphopyridine nucleotide could be demonstrated in cultures of the variant strains after successive transfers in the amino acid medium.

Synthesis of nicotinamide by variants

Tests for nicotinamide and diphosphopyridine nucleotide were made by microbiological methods using a stock dysentery culture requiring either nicotinamide or the coenzyme for growth and a culture of *Hemophilus parainfluenzae*. The latter fails to grow when supplied with nicotinamide but grows well in the presence of the nucleotide.

After a series of transfers in the amino acid medium without added nicotinamide, several representative variant strains were grown in 120 ml. quantities of the same medium for 4 days at 37°C. The cultures were neutralized with sterile sodium hydroxide solution and centrifuged. The cells were resuspended in 20 to 25 ml. of the supernatant. Thus, the proportion of cells to liquid part of the culture was increased. The heavy suspensions of cells were alternately frozen in carbon dioxide-acetone mixture and thawed five times. They next were filtered through sintered glass and the filtrates tested for sterility before use.

Varying amounts of the filtrates were added to tubes of (1) glucose veal-infusion broth and (2) the amino acid medium. The broth was inoculated with *H. parainfluenzae* and the synthetic medium with a dysentery culture. Ap-

TABLE 4

Filtrates of variant cultures support growth of Hemophilus parainfluenzae and Shigella paradyserteriae

5 ML. BASAL MEDIUM* PLUS FILTRATES OF FROZEN AND THAWED CELLS OF:	GROWTH OF <i>H. PARAINFLUENZAE</i> ON ADDITION OF FILTRATE			GROWTH OF <i>S. PARADYSENTERIAE</i> 76 ON ADDITION OF FILTRATE		
	3.0 ml.	2.0 ml.	1.0 ml.	3.0 ml.	2.0 ml.	1.0 ml.
Variant 76.....	+	+	0	+	+	0
Variant 3.....	++	+	0	++	+	0
Variant 8.....	++	+	0	++	+	0
Variant 269.....	++	++	+	++	+	0
<hr/>						
CONTROLS	<i>H. PARAINFLUENZAE</i>			<i>S. PARADYSENTERIAE</i> 76		
DPN 0.03 µg per ml.....	+++			++		
Nicotinamide 0.006 µg per ml.	0			+++		
Nothing added.....	0			0		

* Veal infusion broth was used for *H. parainfluenzae* and the amino acid synthetic medium for *S. paradyserteriae*.

0 = no visible growth, + to +++ indicates light to pronounced turbidity.

DPN = diphosphopyridine nucleotide.

appropriate controls without the filtrates and also with known amounts of nicotinamide and diphosphopyridine nucleotide were also included.

The result is shown in table 4. Culture filtrates of each variant strain supported growth of both *H. parainfluenzae* and *S. paradyserteriae*. Evidently the variant cultures produced diphosphopyridine nucleotide or something physiologically equivalent to it. From these tests it appears probable that nicotinamide is synthesized by the variants.

Biochemical and agglutination tests of variants

After serial transfer in the amino acid medium each variant strain was tested with respect to the salient characteristics for identification of dysentery bacilli. In glucose, mannitol, sucrose and lactose broth, no difference in fermentative ability between the variants and the stock cultures was observed. Also, the

variants were agglutinated by antiserum to approximately the same titer as the original stock cultures.

Reversion

The variant strains, after serial transfer in the amino acid medium without nicotinamide, were again grown in the presence of nicotinamide to see if they would revert to their original status. Tests were made in different media: (1) daily transfers on veal infusion agar at 37°C. for 2 weeks plus an additional period of 150 days in the ice box with several more agar transfers in this interval, (2) daily transfers for 7 days in veal-infusion broth at 37°C. with several additional transfers at weekly intervals for 1 to 2 months, (3) daily transfers for 7 days at 37°C. in the amino acid medium plus 0.1 μ g nicotinamide per ml. with additional transfers at weekly intervals for 1 to 2 months, (4) transfers every 1 or 2 days in 1 per cent glucose veal-infusion broth for approximately 50 transfers.

The first three methods failed to produce any evidence of reversion; the variants developed to the same extent as before in the amino acid medium as shown by colorimeter tests of turbidity. Perhaps in these cases the number of daily transfers was insufficient. With the fourth procedure a loss of ability to grow in the amino acid medium became evident. These cultures were tested for growth in the synthetic medium after every seventh transfer in glucose broth. A distinct lessening of the amount of growth was noted after the 7th, 28th and 35th such transfers of variants 3, 269, and 8, respectively. Once this change had occurred, usually only a few additional glucose broth transfers were needed to bring the variant approximately to the point where its behavior was similar to the original culture.

SUMMARY

From four laboratory stock cultures of dysentery bacilli requiring nicotinamide for growth, variants were obtained capable of growth in successive transfers without added nicotinamide. These variants could be obtained rather readily in an amino-acid-glucose-salt medium but not when ammonium phosphate replaced the amino acids.

The variants were obtained both by serial transfers in the amino acid medium in the presence of decreasing amounts of nicotinamide and by bulk inoculation of the amino acid medium followed by subsequent transfers without added nicotinamide, either immediately or after a period of incubation.

In the bulk inoculation method the proportion of such variants to the total number of viable cells increased during incubation of the tube receiving the heavy inoculum.

The variants, once established in successive transfers, never developed as luxuriantly without nicotinamide as in the presence of optimum amounts of this accessory factor.

Culture filtrates of the variants, after growth in medium without nicotinamide, supported growth of an original stock dysentery culture and of *Hemophilis parainfluenzae*. Apparently nicotinamide and coenzyme or something physiologically equivalent are synthesized by the variants.

The common sugar fermentation tests and agglutination by immune serum did not change with adaptation to growth without added nicotinamide.

The variants reverted in several instances to their original nicotinamide requirement during successive transfers in glucose meat-infusion peptone broth.

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STREPTOCOCCUS ALLANTOICUS AND THE FERMENTATION OF ALLANTOIN

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Only a few kinds of bacteria are known to satisfy their energy requirements by decomposing single nitrogenous compounds under anaerobic conditions. These are *Clostridium tetanomorphum* and *C. cochlearium* (Barker, (1937, 1939)) which ferment glutamic acid, *C. aciduri* and *C. cylindrosporum* (Barker and Beck (1941, 1942)) which ferment uric acid and some other purines, an unnamed *Clostridium* species which ferments alanine, serine and threonine, and an unnamed obligately anaerobic coccus (Cardon, (1942)) which ferments glycine. *C. botulinum* (Clifton (1940)), *C. sporogenes* (Hoogerheide and Kocholaty (1938)) and *C. tetani* (Clifton (1942)) may also be able to live by the fermentation of single amino acids, though the affirmative evidence is not complete. The present paper reports a new type of energy-yielding anaerobic process, the fermentation of allantoin, and describes the causative organism, *Streptococcus allantoicus*, which appears to be a new species.

ISOLATION AND CHARACTERISTICS OF STREPTOCOCCUS ALLANTOICUS

An enrichment medium containing 0.4 per cent allantoin, 1 vol. per cent yeast autolysate, 0.1 per cent K_2HPO_4 , 0.01 per cent $MgSO_4 \cdot 7H_2O$, tapwater, pH 7.4 was inoculated heavily with black mud from the shore of San Francisco bay and was incubated at 28°C. in a completely filled glass-stoppered bottle. Within four days a large number of streptococci had developed and the allantoin had been decomposed with the formation of ammonia and urea. After one transfer, the organism was isolated by the shake culture method, using the same medium supplemented with agar and 0.02 per cent $Na_2S \cdot 9H_2O$.

The isolated organism (*Streptococcus allantoicus*) is a rather large, gram-positive, nonmotile streptococcus (fig. 1). The chain length and the size of individual cells vary considerably with the conditions of growth. Surface colonies, like those of many streptococci, are small (1-2 mm. diam.), low dome-shaped, circular, entire, moist, and greyish white. Liquid cultures are at first uniformly turbid but within 2-3 days the cells sediment, leaving a clear supernatant medium. With glucose as a substrate the organism produces a thick polysaccharide slime; with fructose very little slime is formed, while with allantoin there appears to be none. Like other lactic acid bacteria, the organism is catalase-negative.

S. allantoicus is a facultative anaerobe. As substrates for anaerobic growth it can use allantoin, glucose, fructose, galactose, sucrose, lactose, maltose, salicin, arabinose, xylose, raffinose, inulin, mannitol and sorbitol. All these compounds with the exception of mannitol are utilized rapidly, maximum growth being at-

tained in 24–36 hours, other conditions being favorable. Mannitol is decomposed more slowly under anaerobic conditions; in tube cultures of semisolid mannitol medium exposed to air, growth occurs first at the surface and is followed about a day later by subsurface growth. The behavior is such as to suggest that a special adaptation is required for the anaerobic utilization of mannitol. Glycerol is utilized in the presence, but not in the absence, of oxygen. With allantoin, almost the reverse is true. Anaerobically, colonies 0.5 mm. in diameter are formed within 24 hours, while in the presence of oxygen the colonies never exceed 50 μ in diameter when they stop growing after 3–4 days incubation. With glucose on the contrary the growth rate is almost independent of oxygen.

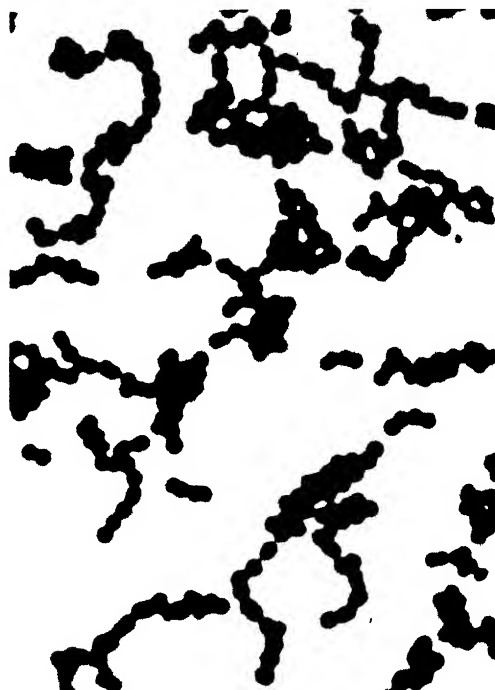


FIG. 1. *S. ALLANTOICUS* FROM A TWO-DAY-OLD STAB CULTURE OF ALLANTOIN-YEAST EXTRACT MEDIUM
Gentian violet. 1000 \times

No explanation for the anomalous behavior of allantoin in relation to oxygen can as yet be offered. Starch and rhamnose are not attacked under any conditions.

In addition to a fermentable or oxidizable substrate, *S. allantoicus* requires small amounts of yeast extract or similar material. A supplement of 100 mg. Difco yeast extract per 100 ml. allows maximal growth. However, yeast extract, alone (i.e., in the absence of a fermentable substrate) or with 1 per cent tryptone, permits only very slight growth, accompanied by a small ammonia production (0.01 mg. NH_3 -N/ml.). Peptone (1 per cent) and beef extract (0.3 per cent) together markedly inhibit growth in allantoin or glucose media.

These observations indicate that the nitrogenous compounds present in yeast extract, peptones or beef extract cannot replace allantoin as a good energy source. The further nutritional requirements of the organism will be considered in some detail in a later paper. At present it need only be mentioned that one of the essential constituents of yeast extract is biotin.

The optimum temperature for growth is close to 32°C.; at this temperature, growth becomes macroscopically visible in a lightly inoculated yeast-extract glucose medium in about four hours. The bacteria do not develop above 36.5°C.

Unlike many streptococci, *S. allantoiacus* is not acid-tolerant. Growth is most rapid at pH 7-8. pH 6 is about the lower limit for initiation of growth. However, when the fermentation of glucose is started in a neutral medium the pH will drop to 5.4, at which reaction cultures become sterile within a day or two. To completely ferment 1 per cent glucose, a strongly buffered medium must be used and in addition it must be neutralized two or three times during the incubation. A suitable buffer is K_2HPO_4 , which can be tolerated to a concentration of about 2 per cent although above 1.5 per cent the growth rate is decreased.

As an aid to classification, the growth of *S. allantoiacus* on a number of special media was tested. In plain milk there was little growth and no visible change in appearance. Litmus added to milk was not reduced. Rapid and abundant growth occurred on aerobic plates of the following agar media: nutrient blood, yeast-extract glucose, yeast-extract glucose blood, yeast-extract glucose calcium-carbonate. No haemolysis occurs in nutrient blood agar, though in media containing added glucose faint greenish zones develop slowly. In a yeast-extract glucose semisolid medium containing 6.5 per cent NaCl, growth is slow but normal in amount.

Since on the basis of the above characteristics and the metabolic activities to be described below, the organism could not be identified with any previously described streptococcus, it is tentatively proposed to create a new species, *Streptococcus allantoiacus*. A decision as to the validity of this species must be left to future students of the streptococci.

CARBOHYDRATE FERMENTATIONS

The fermentations of glucose and fructose were studied to learn whether *S. allantoiacus* belongs to the homofermentative or heterofermentative group of lactic acid bacteria.

The products of glucose fermentation (table 1) were determined in a medium of the following composition: Speakman's salts 1 g., $(NH_4)_2SO_4$ 1 g., phenol red indicator 30 drops of a 0.05 per cent solution, anhydrous glucose or fructose 30 g., Difco yeast extract 0.5 g., biotin 0.1 γ , distilled water to 900 ml. After autoclaving at pH 6.5, 100 ml. of a sterile 9 per cent K_2HPO_4 solution and 10 ml. of a 1 per cent $Na_2S \cdot 9H_2O$ solution were added, to give a medium of pH 8. Oxygen was excluded by an Oxsorbent (acid chromous sulfate) seal. The cultures were incubated for 3 days at 28°C., during which time they were twice neutralized to pH 7.6 with sterile CO_2 -free NaOH.

Since most of the fermentation products were determined by common analyti-

cal methods, only the following comments will be made. The d-form of lactic acid was identified by the water of crystallization and the negative optical rotation of its purified zinc salt. Glycerol was determined directly on the fermented medium by the periodate oxidation method of Voris, Ellis and Maynard (1940); the recorded data are corrected for residual sugar but since certain constituents of the yeast extract reduce periodate the results tend to be high. "Cell material" represents the insoluble mixture of cells and capsular polysaccharide separated by centrifugation; the organic (ash-free) part of this material was assumed to contain 48 per cent carbon. The polysaccharide which makes up the greater part of the "cell material" is a dextran, yielding only glucose on hydrolysis.¹

The glucose fermentation is neither typically homofermentative nor heterofermentative. The small yield of carbon dioxide (no visible gas) indicates a homofermentative type of metabolism. On the contrary the relatively low yield of lactic acid and moderately high yields of volatile acids and alcohol are charac-

TABLE 1
Fermentation of glucose

PRODUCT	MM/100 MM FERMENTED GLUCOSE
d-Lactic acid.....	106
Acetic acid.....	36.0
Formic acid.....	23.9
Carbon dioxide.....	9.7
Ethyl alcohol.....	27.6
Glycerol.....	<0.6
Cell material (as carbon).....	71.2
Carbon recovery.....	92.1%
Available hydrogen recovery*.....	93.0%

* See Barker (1936).

teristic of a heterofermentative lactic acid fermentation. However, Gunsalus and Niven (1942) have recently shown that the production of volatile acids and alcohol is not restricted to heterofermentative species but may be obtained with typically homofermentative streptococci provided the medium is maintained at or near a neutral reaction, as was done in the present experiments. A distinctive feature of the glucose metabolism is the rather large production of dextran. Only *S. liquefaciens* is known to form a comparable amount of dextran from glucose (Gunsalus and Niven (1942)).

Mannitol could not be detected by the periodate oxidation method among the products of fructose fermentation. Since all heterofermentative lactic acid bacteria give large yields of mannitol from fructose, *S. allantoicus* evidently does not belong in this group.

¹ The author is in debt to Dr. W. Z. Hassid for identifying the polysaccharide as a dextran.

FERMENTATION OF ALLANTOIN

For determining the products of allantoin fermentation the following medium was used: Speakman's salts 1 g., biotin 0.1 γ , Difco yeast extract 0.3 g., K_2HPO_4 0.9 g., allantoin 10 g., $Na_2S \cdot 9H_2O$ 0.1 g., pH 6.8, distilled water to 1 liter. Since allantoin decomposes extensively during autoclaving it was sterilized separately by filtration through a Seltz filter after being dissolved as quickly as possible in water at 90°C. The inoculated medium was protected from oxygen by an Oxsorbent seal. Incubation was at 28°C. for 4–6 days.

During the fermentation there was no visible gas production; the pH increased only about 0.2 unit. The allantoin was mostly decomposed in the first 24 hours.

The initial allantoin was calculated on the basis of Kjeldahl nitrogen determinations, after correcting for nitrogen in the yeast extract. The residual allantoin after fermentation was very small and was determined by the colorimetric method of Young and Conway (1942). Ammonia was estimated by a five-minute steam distillation in a Kirk (1936) micro-Kjeldahl still; suitable corrections were made for ammonia produced by decomposition of urea and allantoin during the distillation. For estimating urea the van Slyke manometric urease method (Peters and van Slyke (1932)) was used. Carbon dioxide was also determined by the van Slyke manometric method. Formic and acetic acids were determined by mercuric chloride reduction and titration, respectively. The carbon and nitrogen of the cell material was obtained by centrifuging, washing and drying the cells at 100°C. and assuming that the dry cells contain 50 per cent carbon and 10 per cent nitrogen.

Preliminary experiments showed that only about 85 per cent of the nitrogen and 70 per cent of the carbon of the fermented allantoin could be accounted for as ammonia, urea, carbon dioxide, acetic and formic acids. Further investigation showed that all of the missing nitrogen and most of the carbon is present in oxamic acid, the monoamide of oxalic acid, a compound not previously observed as a product of bacterial metabolism.

The first indication of the presence of oxamic acid was the formation of oxalic acid when the fermented medium was boiled for a half hour at pH 2. It was then found that during acid hydrolysis oxalate and ammonia are formed in equimolecular quantities; the rate of oxalate formation in 0.5 N HCl at 100°C. was approximately the same in the fermented medium as in a solution of synthetic oxamic acid. Finally, the beautifully crystalline calcium oxamate was obtained from a concentrated sample of the fermented medium by addition of calcium acetate. Free oxamic acid could also be isolated by ether extraction from acid solution. The free acid was prepared for analysis by being twice recrystallized from water, washed with alcohol and ether and dried *in vacuo* at 50°C. Analytical data are given in table 2. The data proved that the isolated compound is oxamic acid.

Quantitative estimation of oxamic acid was carried out by determining the oxalic acid formed by complete acid hydrolysis. 4 ml. of fermented medium and 4 ml. 4 N HCl were placed in a 15 ml. centrifuge tube and heated in an

Arnold steam sterilizer for 45 minutes. After cooling, 0.5 ml. of a saturated sodium acetate solution and several drops of brom cresol green indicator were added. The solution was then adjusted to pH 5 with alkali and 1 ml. of 5 per cent calcium chloride solution was added to precipitate calcium oxalate. The oxalate was determined by the usual acid permanganate titration.

After finding oxamic acid, 99–100 per cent of the nitrogen of the fermented allantoin could be accounted for, but about 8 per cent of the carbon was still missing. Tests were therefore made for various non-nitrogenous substances, particularly C_2 compounds, that might conceivably be formed from allantoin. It was found that some substance (or substances) was present in the fermented medium which yielded oxalic acid by alkaline permanganate oxidation (Evans and Adkin (1919)) in almost exactly the amount required to account for the missing carbon. About 10 per cent of the oxalate apparently comes from lactic acid (determined by the Friedemann and Graeser (1933) method). The remaining 90 per cent of the oxalate is derived from an, as yet unidentified, compound which appears to be a non-volatile acid since it is extractable with ether

TABLE 2
Identification of oxamic acid

PROPERTY	ISOLATED COMPOUND	SYNTHETIC OXAMIC ACID
Melting point (with decomposition) ..	205° (uncorr.)	204° (uncorr.)*
Acid equivalent weight	89.5	89.0
Percentage nitrogen	15.3	15.7
Ratio: NH_2 /oxalic acid	1.00	1.00

* Oelkers (1889) gives the melting point of oxamic acid as 210°. Our oxamic acid was prepared by alkaline hydrolysis of oxamethane (the ethylester of oxamic acid) and was twice recrystallized.

from an acid but not a neutral solution. However, the rate of acid-ether extraction is very low, being only 35 per cent complete after 40 hours and 90 per cent complete after 144 hours. The compound could not be precipitated from dilute aqueous solution with either barium or basic lead acetate. The available evidence suggested the compound might be glycollic acid, but all attempts to isolate this substance were unsuccessful. Nevertheless, in making the carbon and oxidation reduction balances of table 3, the unidentified product is assumed to be glycollic acid.

Five C_2 compounds could be proved to be absent from the fermented medium, namely, ethanol, acetaldehyde, glyoxylic acid, ethylene glycol and oxalic acid. Ethanol and acetaldehyde were eliminated by the absence from a neutral distillate of any substance capable of reducing acid dichromate; glyoxylic acid was eliminated by a negative Rimini-Schryver reaction (Young and Conway (1942)); since periodate was not reduced, ethylene glycol could be excluded; no oxalate could be precipitated with calcium except after either acid hydrolysis or alkaline permanganate oxidation.

Typical data on the products of allantoin fermentation are given in table 3. Yields obtained in other experiments were very similar. In particular it is significant that the ratio of ammonia to urea is constant under the experimental conditions. This suggests that ammonia is formed directly from allantoin or some intermediate rather than by hydrolysis of urea. This interpretation was confirmed by showing that cell suspensions of *S. allantoinicus* are unable to decompose urea.

DISCUSSION

The above experiments show that *S. allantoinicus* is able to develop readily on a medium containing allantoin as the main organic constituent. Since growth is negligible when allantoin is omitted and since allantoin undergoes a typical fermentative decomposition, there can be no doubt that this process provides energy for the vital activities of the organism. As mentioned in the introduction,

TABLE 3
Fermentation of allantoin

PRODUCTS	MM/100 MM FERMENTED ALLANTOIN
Ammonia.....	226
Urea.....	62.3
Oxamic acid ..	44.8
Carbon dioxide.....	168
Formic acid.....	9.4
Acetic acid.....	14.8
Glycollic acid (?).....	13.8
Lactic acid.....	1.5
Cell carbon.....	13.1
Nitrogen recovery.....	99.5%
Carbon recovery.....	101.2%
Available hydrogen recovery.....	101.8%

several other bacteria have previously been shown to obtain energy by the anaerobic decomposition of nitrogenous compounds, but *S. allantoinicus* is the first streptococcus, or more generally, the first organism belonging to the large group of lactic acid bacteria, which is known to live by such a process.

The fermentation of allantoin is of some biochemical interest because it is one of very few fermentations involving a substrate with only two adjoining carbon atoms. Allantoin is a derivative of the diureide of glyoxylic acid, a C_2 compound; therefore the fermentation of allantoin may be thought of as a fermentation of glyoxylic acid. Theoretically, the reduction of glyoxylic acid could produce glycollic and acetic acids, while by oxidation oxalic and formic acids and carbon dioxide could be formed. Actually, several of these theoretical possibilities are realized: acetic and formic acids and carbon dioxide² are formed;

² Note that the yield of carbon dioxide is greater than could come from the urea residues of allantoin.

oxalic acid appears in the form of its amide, oxamic acid; and glycollic acid may also be a product, though the evidence is not complete.

The only product (other than cell material) not theoretically derivable from glyoxylic acid by reduction or by oxidation and decarboxylation is lactic acid. The very small quantity of this C_3 compound may well arise by a secondary fermentation of cellular carbohydrate.

The anaerobic decomposition of allantoin described here is to be contrasted with the aerobic decomposition brought about by *Corynebacterium ureafaciens* (Krebs and Eggleston (1939)) and other soil bacteria (Liebert (1909)). The latter process goes on only in the presence of oxygen and results in the formation of almost 2 moles of urea per mole of allantoin as compared with 0.6 mole urea in the anaerobic process. Also little or no ammonia is formed by the aerobic bacteria. For these and other obvious reasons the aerobic and anaerobic decompositions of allantoin need never be confused.

SUMMARY

The isolation and characteristics of a possibly new species of streptococcus, *Streptococcus allantoicus*, are described. This organism carries out a modified homofermentative lactic acid fermentation of sugar and is also able to develop anaerobically with allantoin as a carbon and energy source. The fermentation of allantoin results in the formation of ammonia, urea, carbon dioxide, formic, acetic, lactic and oxamic acids, and possibly glycollic acid.

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THE OCCURRENCE AND BACTERIOLOGICAL CHARACTERISTICS OF *S. MARCESCENS* FROM A CASE OF MENINGITIS

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Acute cerebro-spinal meningitis has been attributed to a large variety of bacteria, but no previous report incriminating *Serratia marcescens*, as the etiological agent has been found, in the literature.

There has long existed the impression, that *S. marcescens* is a nonpathogenic organism and it has been used frequently for various bacteriological studies, especially those dealing with the mechanism of the spread of droplet infection. That *S. marcescens* is pathogenic for animals has been shown by Bertarelli (1903), who found that white mice and white rats were killed by the intraperitoneal injection of small amounts of the culture while guinea pigs and rabbits were less susceptible. From the sputum of a case of pneumonia Aitoff, Dion, and Dobkevitch (1936) isolated a strain of *S. marcescens* which was highly pathogenic for mice, rats, guinea pigs, and rabbits. They concluded that while *S. marcescens* is usually saprophytic it may occasionally be pathogenic. Rosahn and Hu (1933) found that cultures of *S. marcescens* were pathogenic for rabbits and mice, when injected either intracerebrally, intraperitoneally, intravenously, or intratesticularly.

The pathogenicity of *S. marcescens*, for man, is indicated by the isolation of this organism from two different samples of spinal fluid from the following case:

A colored soldier 21 years old who contracted syphilis in 1937, received anti-syphilitic treatment, at intermittent intervals. In July 1941 a lumbar puncture was performed as a routine diagnostic procedure, at which time the spinal fluid showed 3 cells per mm.³, no increase in globulin, positive Wassermann reaction, and a colloidal gold curve of the paretic type (5442211000). Antisyphilitic treatment was continued, and on February 14, 1942 the lumbar puncture was repeated. The spinal fluid at this time showed 5 cells per mm.³, globulin slightly increased, Wassermann reaction and colloidal gold curve the same as that noted previously. The patient at this time complained of a "cold" and cough, as well as pain, at the site of the lumbar puncture wound. The pain, however, did not differ materially from that noted following previous lumbar punctures. On February 17th, three days following the lumbar puncture, the patient reported that his "cold" and cough were worse and complained of a severe chill, fever and vomiting, followed by a sudden, intense, deep seated headache and nuchal rigidity. The patient was admitted to the Station Hospital, Fort Belvoir, Va. at 10:00 P.M. February 17, at which time he was restless and complained of intense headache and backache. His temperature was 102°F., pulse and respiration 100 and 24 per minute respectively. Physical examination revealed well marked congestion of the throat, positive Kernig sign and positive Brudzinski. No

local inflammatory reaction was noted at the site of the lumbar puncture, although tenderness was elicited, locally, upon deep pressure. A lumbar puncture was performed shortly after his admission to the hospital and 20 ml. of a cloudy spinal fluid, which was under increased pressure, was removed. The spinal fluid, which did not coagulate on standing contained 2100 cells per mm.³, 92 per cent of which were polymorphonuclear cells, and 8 per cent lymphocytes. The globulin was increased and the sugar was 50 mg. per 100 ml. No bacteria were found in smear preparations made directly from the spinal fluid nor in the sediment obtained after centrifuging. Examination of the blood showed 11,400 leucocytes, 82 per cent of which were polymorphonuclear cells. A blood culture taken shortly after his admission was sterile. The lumbar puncture was repeated February 18th at which time the spinal fluid contained 1700 cells per mm.³, globulin was increased, sugar 92 mg. per 100 ml. No bacteria were found, in either the smear made directly from the spinal fluid or in the sediment after centrifuging. The patient rapidly improved and the spinal fluid obtained February 28th was clear, with a cell count of 25 cells per mm.³, 30 per cent of which were polymorphonuclear cells and 70 per cent lymphocytes. The patient continued to improve and was discharged from the hospital as cured May 8, 1942.

From the specimens of spinal fluid, obtained on February 17 and 18, planted on human serum agar, there was noted, after 18 to 24 hours incubation at 37°C., a profuse growth of confluent, grayish and faintly pink colonies which, when exposed to daylight, assumed a deep red color. Upon microscopic examination, small, actively motile organisms were found which stained with the usual aniline dyes and were gram-negative.

Cultivation

On beef infusion agar, pH 7.4, the colonies, of the primary culture, appeared as sharply defined, rounded, opaque, smooth, shiny, soft, convex, elevated colonies, colored grayish or faint pink when grown in the dark, at either 37 or 20°C. When exposed to daylight the colonies assumed a deep red or pink color which did not, however, diffuse through the agar. In beef infusion broth, pH 7.4, the growth was rapid, resulting in a uniform turbidity. A red ring was noted, at the surface of the media and a heavy grayish white sediment, was present at the bottom. Gelatin was liquefied and the liquefied portion assumed a deep red color. On coagulated serum medium liquefaction occurred along the line of inoculation, which assumed a deep red fuchsin color with a metallic luster. On potato, the growth was luxuriant, at first dirty gray in appearance, later becoming dark red. Litmus milk was acidified and coagulated but was not digested. On human or rabbit-blood agar complete hemolysis was noted for several millimeters beyond the margin of the colony. Growth on the different media was more profuse at 20 than at 37°C., while coloration of all media, except coagulated serum occurred only when the culture was exposed to sunlight.

Biochemical reaction

Nitrates were reduced to nitrites, indole was not produced, and an odor of trimethylamine, most marked in the broth, was observed in the various culture

media. Acid, but no gas, was produced in mannitol, galactose, sucrose, maltose, lactose, and glucose, while neither acid nor gas were produced in xylose, raffinose, arabinose, or inulin. The pigment was soluble in water, chloroform, decinormal hydrochloric acid and decinormal sodium hydroxide with the production of a deep red color. In 95 per cent and absolute ethyl alcohol, ether, acetone, xylene, methyl alcohol, carbon disulphide and carbon tetrachloride the color of the pigment varied from a faint to a deep pink shade. The pigment, was adsorbed on the surface of asbestos filters.

Spectrophotometric studies were made of the pigment produced by this culture. The culture grown on beef infusion agar, pH 7.4, was transferred to the various solvents and after standing at 20°C for 24 hours the solutions were centrifuged and the absorption bands of the supernatant fluid were determined spectrophotometrically. The results obtained are presented in table 1. It is evident from table 1 that the fraction which absorbed the maximum wave-length of 532 to 550 was soluble in all of the solvents except ether, in which only a trace

TABLE 1
Wavelength of maximum absorption of pigment of S. marcescens

SOLVENT	WAVE-LENGTH	SOLVENT	WAVELENGTH
Acetone	532	Ether	460 and 536
Petroleum ether	534	Ethyl alcohol absolute	466 and 536
Amyl alcohol	534	Methyl alcohol	470 and 536
Xylene	536	Sodium hydroxide N/10	494, 536, and 572
Hydrochloric acid N/10	538		
Water	540		
Benzene	540		
Chloroform	540		
Carbon tetrachloride	544		
Carbon disulphide	550		

of the 536 constituent was present; while fractions having an absorption band in the 460 to 470 wavelength were soluble in ether, ethyl alcohol, and methyl alcohol. The differences in the absorption curve of the pigments soluble in chloroform, ether, and decinormal sodium hydroxide are presented graphically in figure 1.

This culture when grown in broth at 20 or 37°C. for 24 hours or longer produced a hemolysin which, in amounts of 0.1 ml. or more, hemolyzed 0.2 ml. of a 2 per cent suspension of washed blood cells of human, rabbit, guinea pig, mouse, rat, horse, or hamster. This hemolysin was inactivated by filtration through a Seitz filter or by heating for 30 minutes at 56°C. Filtrates of the culture were not toxic for either mice or guinea pigs.

Colonial variation

Bacterial dissociation manifested itself by the appearance of nonchromogenic colonies and by colonial variation. The mutation from chromogenic to nonchromogenic colonies was not observed in all generations. Nonchromogenic colonies transplanted to the same culture medium yielded numerous nonchromo-

genic colonies as well as numerous chromogenic colonies. The nonchromogenic colonies were moist, smooth, elevated, and when emulsified in physiological salt solution yielded a uniform cloudiness. No significant difference was noted in the virulence of the nonchromogenic and chromogenic colonies for mice.

Striking variations from the original culture (fig. 2) were noted in the colonial characteristics of cultures prepared from the blood of mice inoculated with this culture. Some of these colonies showed an elongated center from which there projected at right angles thick fingerlike processes resembling a package of fire crackers (fig. 3), other colonies were large, flat, slightly elevated, with linear colonies radiating from a center giving rise to a medusoid appearance (fig. 4). Some colonies resembled a bursting bomb while others had a large thick center

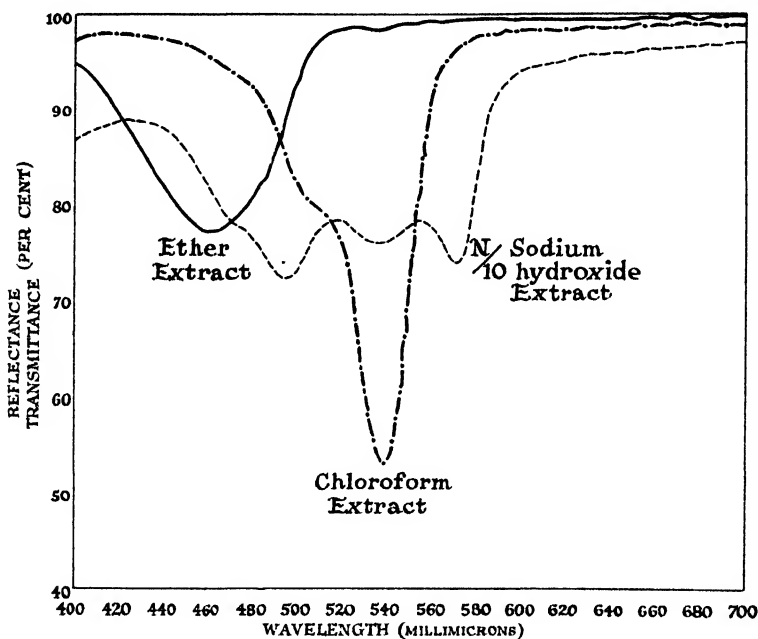


FIG. 1. ABSORPTION BANDS OF PIGMENT OF *S. MARCESCENS*

from which there radiated lobose projections, giving rise to a cocks-comb appearance (fig. 5). Despite variations in colony morphology these colonies were chromogenic, soft, smooth, moist, and when emulsified in physiological salt solution gave a uniform turbid suspension. These changes in colonial characteristics have thus far been observed only on cultures made from blood of infected mice, and when transplanted to the usual culture media the colonies assumed the colonial appearance of the original culture.

Pathogenicity

Broth cultures grown at 20 or 37°C. for from 18 to 24 hours were pathogenic for white mice, white rats, hamsters (*Cricetus auratus*), turtles (*Terrapene carolina*) and rabbits but not for guinea pigs. White mice injected intraperitoneally

with 0.05 ml. or more of an 18-hour-old broth culture died in from 4 to 5 hours while mice injected in the same manner with similar amounts of culture and an equal amount of 6 per cent mucin died in from 2 to 3 hours. Hamsters (*Cricetus auratus*) died in from 6 to 12 hours following the intraperitoneal injection of 0.5 ml. of an 18-hour-old broth culture. Turtles (*Terrapene carolina*), injected intraperitoneally with 1.0 ml. of a broth culture died in 48 hours. Rabbits died in 12 hours after the intravenous injection of 1.0 ml. of the culture. Smears prepared from the peritoneal exudate of mice 30 minutes after the injection of



Fig. 2



Fig. 3



Fig. 4



Fig. 5



Fig. 6

FIGS. 2-6

0.05 ml. of a broth culture showed a moderate number of polymorphonuclear cells and large numbers of gram-negative bacilli many of which had a well defined capsule (fig. 6). Smears made at 30-minute intervals showed a decreasing number of polymorphonuclear cells and a marked increase in the number of bacteria, only a few of which were phagocytosed.

To study the pathogenicity of this organism further, cultures were made at intervals from the blood of the tail of mice, injected intraperitoneally, with varying amounts of the broth culture. Blood was collected at 5-minute intervals,

for the first 15 minutes and at 15-minute intervals, for the following two hours. In mice injected with 10^{-4} , positive blood cultures were obtained in several mice 15 minutes following intraperitoneal inoculation while all animals yielded positive blood cultures 45 minutes after inoculation. In mice injected with 10^{-3} several showed positive blood cultures 5 minutes after inoculation while all animals gave a positive blood culture 15 minutes after inoculation. With 10^{-2} or larger doses positive blood cultures were obtained in all of the inoculated mice 5 minutes after infection. The number of colonies bore a direct relationship to the dose of inoculum, and were most numerous in the blood collected within the first 15 minutes after inoculation, after which time the number of colonies decreased. In mice receiving 0.1 ml. of the broth culture the colonies cultured from the blood were too numerous to count.

Pathology

At necropsy there was noted, in mice which died following the intraperitoneal injection of this culture, marked congestion of the vessels in the subcutaneous tissue of the abdomen. The peritoneum was somewhat reddened and the peritoneal cavity contained an excess of a cloudy, grayish fluid which had a mucous-like consistence. The serosal surface of the intestines was reddened and the vessels injected. The spleen was somewhat larger than normal, dark red in color, while the capsule was smooth and matte in appearance. The cut surface was dark red, surface markings were inconspicuous, and the pulp softer than normal. The liver which was of normal size, was dark red and firm. On section the larger vessels were dilated and filled with blood. The kidneys were congested but otherwise appeared normal. The adrenals were congested, but the heart and lungs showed no gross, pathological changes. On microscopic examination the spleen showed extensive areas of hemorrhage throughout the red pulp, with the cells of the Malphigian bodies widely separated by dilated sinuses. In the liver the central veins were dilated and filled with blood and the columns of liver cells were separated by sinuses distended with blood. The Kupfer cells were prominent but the hepatic cells showed no conspicuous changes. The interlobular veins were filled with blood but the bile ducts showed no significant pathological changes. In the kidneys the capillary tufts of the Malphigian bodies and the interlobular vessels were filled with blood. Numerous small hemorrhagic areas were noted throughout the kidney. The epithelial lining of the tubules showed no significant pathological changes. The myocardium showed no conspicuous changes. In the adrenals the capillaries in the zona fasciculata were dilated and filled with blood.

SUMMARY

1. From the spinal fluid of a case of cerebrospinal meningitis, there was isolated an organism which presented the cultural and biochemical characteristics of *Serratia marcescens*.

2. This culture which was pathogenic for mice, rats, rabbits, hamsters, and turtles produced a labile hemolysin.

3. Cultures of this organism showed variations in colonial characteristics as well as in the production of pigment.

4. The pigment produced by this organism consists of fractions which differ in their light absorption characteristics.

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STUDIES ON AGAR CONTAMINATION AS AFFECTING THE STERILIZATION OF CULTURE MEDIA

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Huntoon's hormone agar has been used at the Hooper Foundation for Medical Research, University of California, in the preparation of a bacterial vaccine. This medium will not withstand prolonged heating, and it has been customary to autoclave it in 150 to 200 ml. amounts in Roux culture bottles for 20 minutes at 115°C. This time was later increased to 30 minutes. After autoclaving, the culture bottles were incubated for 3 to 4 days at 37°C. to detect contamination, which was usually found in 2 or 3 per cent of the bottles. After harvesting, the concentrated suspensions, diluted vaccine, and packaged vaccine were subjected to the official sterility test of 7 days incubation at 37°C. According to the results of this test, the vaccine was apparently sterile, but if the incubation was continued for 14 days, some lots of vaccine were found to be contaminated with a spore-forming bacillus. This organism was isolated from the vaccine in various stages of preparation, from the incubated but uninoculated medium, and from various lots of agar used in the preparation of the medium.

Spore counts were made on agar in 2 per cent solutions, heated 5 minutes and 60 minutes in boiling water, and no difference was found in the results obtained. Three samples of American agar had counts of 10,000 to 14,000 spores per gram, and one sample of Japanese agar had a count of 1000 spores per gram.

A study has been made of 23 cultures isolated from the heated samples of the four lots of agar, and 5 cultures from the following sources:

Culture No. A from uninoculated hormone agar autoclaved 30 min. at 240°F. and incubated at 37°C.

Cultures No. B and No. E from formalinized bacterial vaccines

Cultures No. C and No. D from uninoculated double strength hormone agar autoclaved 30 min. at 240°F. and held at room temperature.

A summarized description of these five organisms and the twenty-three pure cultures on page 270 shows the general similarity between the two groups. Cultures isolated from the Japanese agar appear similar to those from the American product. The bacilli are predominantly gram-negative, although sometimes gram-positive preparations may be obtained from cultures not more than 10 hours old at 37°C.

The bacilli prefer aerobic conditions and grow only very slowly anaerobically. The more heat-resistant strains are strictly aerobic. Comparative spore counts made in broth and in agar plates demonstrate the importance of free oxygen in

the medium. A much lower count is obtained by the agar plate method. The subsurface colonies in agar are "pin-point," grow very slowly, and appear not much more than 1 mm. beneath the surface. In broth, however, the organisms

TABLE 1
Summary of characteristics of pure cultures

	CULTURE					GENERAL CHARACTERISTICS OF 23 PURE CULTURES
	A	B	C	D	E	
Morphology	Straight rods 1.7y to 2.5y by 0.7y with subterminal, oval spores, slightly larger in diameter than the rods					Same as A, B, C, D, & E
Motility	+	+	+	+	+	(All motile, generally with peritrichous flagella)
	Peritrichous flagella		Peritrichous flagella			
Gram stain	-	-	-	±	-	7 gram +; 6 gram -; 10 gram ±
Oxygen requirements	Aerobic	Aerobic	Aerobic, facultative	Aerobic, facultative	Aerobic	17 aerobic, facultative; 6 strictly aerobic
Temperature requirements	Optimum temperature 37°C. Good growth at 55°C.					19 facultative thermophiles, optimum temperature 37°C.; 4 mesophiles
Acid from carbohydrates	Growth occurred in all cultures even though no acid produced					
Glucose	-	-	+	±	-	Fermented by 18
Levulose	-	-	+	+	+	Fermented by all
Lactose	-	+	-	-	+	Fermented by 4
Glycerol	-	-	+	+	+	Fermented by all
Indole	Not formed by any culture					Not formed
Liquefaction of gelatin	+	-	+	+	-	21 positive cultures
Litmus milk	Alkaline, peptonization	Alkaline, litmus reduced	Litmus reduced; weak coagulation	Alkaline, peptonization	No change	17—alkaline peptonization, 4—litmus reduced; coagulation
Reduction of nitrates	+	-	+	+	-	18 positive cultures
Growth on potato	Smooth white; potato darkened	Smooth white; potato darkened	Granular white; potato darkened	Wrinkled brown potato; darkened	Smooth yellow	Generally chromogenic forms darkening potato; 4 mesophilic forms fail to grow

grow usually as a pellicle, with the formation of considerable flocculent material, which settles to the bottom of the tubes.

Brewer's thioglycollate medium, recommended for sterility tests by the U. S. Public Health Service, Division of Biologics Control, gives highly erratic results with this group of organisms. The germination of spores in higher dilutions may be delayed for a week or more, or may not occur at all if fewer than 10,000 spores

are present. The most satisfactory medium found so far has been the following broth (A.P.H.A., 1941):

	<i>per cent</i>
Beef extract.....	0.3
Tryptone.....	0.5
Glucose.....	0.1
Skim milk.....	1.0
Adjust to pH 7.0	

In this broth, spores even in small numbers germinate in 1 to 3 days at 37°C.

Two heat-resistance tests have been made on spores of culture A in Huntoon's hormone agar, at temperatures of 105°, 110°, and 115°C., according to the method of Bigelow and Esty (1920). The slope of the thermal death time curve on semi-logarithmic paper (the "z" value, according to Ball (1928)) is unusually steep ($z = 13.0$), which indicates a considerable advantage in autoclaving at high as compared to low temperatures. Temperatures below 105°C. have little lethality value for spores.

Heat penetration tests have been made on different depths of 2 per cent agar in Erlenmeyer flasks and in Roux bottles. It was found that the Roux bottles have a much slower rate of heat penetration than the flasks, requiring 10 minutes longer at 115.6°C. (140°F.) for sterilization. Considerable care should be taken to see that the agar in all flasks and bottles is liquid when steam is turned on in the autoclave. Solid agar requires from 5 to 10 minutes longer to sterilize than does liquid agar, the time increase depending on the depth of medium in the containers.

A thermal death time of 2 minutes at 121°C. in Huntoon's hormone agar was assumed in the calculation of sterilization times and temperatures.

Present indications are that to sterilize contaminated agar in liter Erlenmeyer flasks requires 20 minutes at 121°C. (250°F.) or 35 minutes at 115.6°C. (240°F.). In Roux bottles it is necessary to autoclave for 30 minutes at 121°C. (250°F.) or 45 minutes at 115.6°C. (240°F.). If the agar has solidified before steam is turned on in the autoclave, additional time should be allowed.

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CELLULOSE DECOMPOSITION BY AEROBIC MESOPHILIC BACTERIA FROM SOIL

I. ISOLATION AND DESCRIPTION OF ORGANISMS¹

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A large amount of cellulose finds its way into the soil as the chief component of crop residues, and of natural vegetation. Under normal conditions of temperature and moisture this cellulose disappears almost completely and quite rapidly. There must exist in ordinary soils a vigorous aerobic mesophilic population capable of utilizing cellulose. It has been claimed that many fungi are particularly active in this respect, and although a heavy development of fungi may frequently be observed shortly after the addition of cellulosic materials, they do not usually appear to remain dominant for long. There seems to be no good reason for underestimating the importance of the aerobic bacteria in this process.

Descriptions have been published of a considerable number of aerobic cellulose-organisms, but the information about many of them is scanty. In most cases little biochemical work was carried out, and the cultures were not maintained. To a considerable extent attention has been centered on certain organisms such as *Cytophaga hutchinsonii* that appeared to be specific in the utilization of cellulose. Almost all the aerobic mesophilic cellulose bacteria so far described have been placed in four genera though their classification is far from satisfactory. In the fifth edition of Bergey (1939), twenty-seven species of the genus *Cellulomonas* are listed, four species of the genus *Cellvibrio*, three species of a highly-questionable genus, *Cellfalcicula*, and, in an appendix to the *Spirochaetales*, five species of the genus *Cytophaga*. In addition there are a few other cellulose organisms in sundry other genera. This listing is hardly representative of the forms found in soil. Short curved vibrios are common. Out of 17 new species described by Kalnins (1930), 12 were curved rods of the genus *Vibrio*, 4 belonged to the genus *Bacterium* and one to the genus *Bacillus*. Other vibrios have been described, such as *Vibrio agarliquefaciens* (*Microspira agarliquefaciens*, Gray and Chalmers, 1924) and *Vibrio amylocella* (Gray, 1939). Neither the *Cellulomonas* species nor the *Vibrio* species are in any sense specialized; all use a wide range of carbon and nitrogen sources though not necessarily vigorously. Unspecialized soil cytophagas have also been found (Fuller and Norman, 1943). It seems likely, therefore, that the aerobic cellulose organisms in soil are predominantly versatile organisms that individually may not be very vigorous on cellulose but which are likely to be able to maintain themselves in a heterogeneous soil population that is normally utilizing not pure cellulose alone but a complex substrate of which cellulose is only one constituent.

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The purpose of the work described in these papers was to isolate from soil representative aerobic cellulose bacteria, and to determine their activity quantitatively, not only on filter paper (the standard form of cellulose commonly used as the criterion of cellulose decomposition), but also on cellulose preparations from plant materials, in the absence and presence of other cell-wall constituents.

ISOLATION OF ORGANISMS

Isolations were made from top soil sample of four soils: Clarion silt loam (Prairie), Ames fine sandy loam (Planosol, forest-derived), Fayette silt loam (Gray-Brown podzolic) all from Iowa, and Palouse silt loam (Chernozem) from Washington. The organisms described below were selected for detailed study because of their initial vigor in attacking cellulose and the apparent stability of this property over a period of several months. Other forms were obtained which have not yet been fully examined.

No consistent procedure was followed in isolation and purification. Cellulose dextrin agar described by Fuller and Norman (1942) was found to be extremely helpful. Some of the isolations were made by plating soil suspensions directly on this medium. Others were taken from plain agar plates covered by filter paper moistened with Dubos' solution (1928), transferred to cellulose dextrin agar for purification, tested for cellulose-decomposing ability on filter paper strips partially immersed in Dubos solution, and carried either on cellulose, or, in a few cases, on starch. Purification of some of the forms was a matter of difficulty and was only accomplished by repeated dilution and plating on cellulose dextrin agar, the colonies being picked at an early stage. The fact that most colonies of cellulose-decomposers on this medium are surrounded by a halo was of considerable assistance.

DESCRIPTION OF ORGANISMS

The following appear to be new species:

Pseudomonas ephemerocyanea n. sp.

Ephemerocyanea is from the Greek *ephēmeros* and *cyaneos* meaning "short-lived blue."

Morphology. *Vegetative cells:* Straight to slightly bent rods with rounded ends, 2.2–2.8 x 0.3–0.4 microns, arranged singly. *Spores:* Absent. *Motility:* Present, one to three polar flagella. *Staining:* Gram negative.

Cultural characteristics. *Gelatin stab:* Growth and liquefaction. *Starch agar slant:* Heavy, gelatinous, tan to light brown growth. The color deepens to brown in old cultures. *Litmus milk:* No visible growth. *Indole:* Negative. *Nitrite formation:* Nitrate is reduced to nitrite. *Diastase:* Positive. *Carbohydrates:* Glucose, lactose, maltose, galactose, arabinose, and to a lesser extent, xylose, are rapidly attacked. *Polysaccharides:* Cellulose, cellulosan, water-insoluble and water-soluble cellulose dextrans and pectin are readily utilized. Gum arabic and calcium gluconate are only slowly utilized. *Filter paper strips:* Strips of filter paper in mineral nutrient medium may be disintegrated at the

surface of the solution in 24 hours; however, this usually takes 36 hours. Transfers made from thirty-day-old starch agar slants fail to cause fragmentation of the cellulose strips until 4 or more days. The disintegrated region at the liquid-air interface is soft and pulpy and turns light brown after an initial short period during which it may be violet or blue in color. The cellulose below the surface is only slowly decomposed, becoming light brown and pulpy in about 10 to 15 days. The solution becomes turbid as the cellulose is attacked. *Nitrogen*: Peptone, yeast, nitrate, and ammonia are used. *Oxygen*: Highly aerobic. *Temperature*: 22–35°C. *Habitat*: Soil.

Colony characteristics. Starch: Pin-point colonies appear on starch agar medium in 3 days. In 5 days, surface colonies, 1 to 2 mm. in diameter, have a chalk-white color that soon takes on a tan cast. The raised colony has a glistening luster, is smooth, and has an edge that is smooth and entire. Subsurface colonies are small and angular. After 20 days the surface colonies are 3 to 4 mm. in diameter. *Water-insoluble dextrin*: Colonies on water-insoluble dextrin agar appear in 4 or 5 days, and are pin-point in size. Immediately upon the appearance of a colony a distinct enzymatic zone extending $\frac{1}{2}$ to $1\frac{1}{2}$ mm. away from the edge of the colony may be seen. The zone grows as the colony grows. In 7 days the chalk-white colony, that has a convex elevation, is smooth edged and about 1 mm. or less in diameter. After 20 days the colony still looks much the same, but generally grows to a size of about 2 to 3 mm.

Pseudomonas lasia n. sp.

Lasia is from the Greek *lasios*, woolly or shaggy.

Morphology. Vegetative cells: Short, slender rods with rounded ends, 1.2–2.0 x 0.5–0.6 microns. Generally singly; sometimes in chains. *Spores*: Absent. *Motility*: Present. Singly polar flagellum. *Staining*: Gram negative.

Cultural characteristics. Gelatin stab: Thin growth occurs but no liquefaction. *Starch agar slant*: White growth turning to pale yellow in old cultures. *Litmus milk*: Reduction at the bottom of the tube but no peptization, curd, or change in reaction. *Indole*: Negative. *Nitrate*: Nitrate is reduced to nitrite. *Diastase*: Positive. *Carbohydrates*: Glucose, xylose, maltose, and starch are readily utilized. Arabinose and galactose and gum arabic are feebly attacked. No acid is formed. *Polysaccharides*: Cellulose, cellulosan, water-insoluble and water-soluble cellulose dextrans, hemicellulose, and pectin are readily attacked. Acid is not formed. *Filter paper strips*: A very decided clouding of the solution is noticed in about 6 days and at the end of 7 days the filter paper becomes limp and rubber-like. Slight shaking causes partial disintegration of the paper at the surface of the liquid. Pigment usually is not produced in 7-day-old cultures. The paper strip swells, becomes light cream to pale yellow in color, and very flexible in about 12 days. Slight shaking causes the cellulose to disintegrate into a pulpy, shredded mass. *Nitrogen*: Peptone, yeast, nitrate and ammonia are used. *Oxygen*: Aerobic. *Temperature*: 22–35°C. *Habitat*: Soil.

Colony characteristics. Starch: Colonies growing on starch agar appear in 3 to 4 days, are 1–2 mm. in size, and convex in elevation. They are ivory to pale

yellow in color, have an entire and smooth edge, and are round in shape with a flat surface. Subsurface colonies are slightly irregularly round and look like small woolly balls because of their loose surface. As the colonies become older, they change to a cream color. *Water-insoluble dextrin*: Chalk-white subsurface colonies about 1 mm. in diameter surrounded by cleared zones appear in 4-5 days. In 10 days the colonies become light cream to pale yellow in color and are about 2 mm. in diameter. The colonies do not spread on the surface, but prefer to grow down into the medium forming irregularly round shapes of loose woolly appearance. Colonies near the surface are convex.

Pseudomonas erythra n. sp.

Erythra is from the Greek *erythros* meaning "reddish."

Morphology. Vegetative cell: Short rods with rounded ends, 1.2-1.5 x 0.2-0.4 microns, usually arranged singly. *Spores*: Absent. *Motility*: Present. Single polar flagellum. *Capsulation*: Present. *Staining*: Gram negative.

Cultural characteristics. Gelatin stab: No growth. *Litmus milk*: No growth. *Indole*: Negative. *Nitrate*: Negative. *Diastase*: Negative. *Carbohydrates*: No growth. *Polysaccharides*: Cellulose and water-insoluble cellulose dextrans are used, the latter to a lesser degree than the former. *Nitrogen*: Yeast and nitrate are used. *Oxygen*: Highly aerobic. *Temperature*: 22-35°C. *Habitat*: Soil.

Colony characteristics. Starch: Starch does not support growth. *Water-insoluble dextrin*: Cellulose dextrin supports growth only feebly. The colonies that appear after 8 to 10 days of incubation all grow beneath the surface of the medium. They are irregular or angular in shape, usually less than 1 mm. in size, and are surrounded by a clear zone 2 to 5 mm. in diameter. The colonies are buff or red-brown in color. No matter how heavily the medium is seeded, only a few colonies appear. *Filter paper strips*: Cellulose shows signs of being attacked in 4 to 5 days by the appearance of red-brown spots on the paper above the surface of the liquid and the solution becomes cloudy. The brown spotted areas enlarge, become viscous on the surface and the paper changes to a tough membrane with a reddish hue. After ten days incubation, the area involved extends 2-5 mm. upward from the level of the liquid. As decomposition progresses, the filter paper becomes pale brown, thin, tough and flexible. The cellulose does not break apart with moderate shaking but may be wound up in a slimy string.

Achromobacter picrum n. sp.

Picrum is from the Greek, *pikros* meaning "sour", "bitter."

Morphology. Vegetative cells: Short, straight rods with rounded ends, 0.9-1.1 x 0.6-0.7 microns, arranged singly. *Spores*: Absent. *Motility*: Absent. *Staining*: Gram negative.

Cultural characteristics. Gelatin stab: Growth and liquefaction. *Starch agar slant*: White growth. *Litmus milk*: No growth. *Indole*: Negative. *Nitrate*: Nitrate is reduced to nitrite. *Diastase*: Positive. *Carbohydrates*: Glucose and starch are vigorously decomposed producing acid and no gas. Lactose, maltose,

galactose, arabinose and xylose are more slowly utilized. *Polysaccharides*: Cellulose, cellulosan, water-insoluble and water-soluble cellulose dextrans, hemicellulose, and pectin are utilized producing acid. The volatile acid is acetic and non-volatile acid is lactic. *Filter paper strips*: The greatest attack on filter paper is at the air-liquid interface, where, after a period of about 7 days, it becomes pulpy and falls apart on gently shaking. Cellulose below the surface of the liquid swells and loses some of its original rigidity. Pigment is not noticeable. *Nitrogen*: Peptone, yeast, nitrate, and ammonia are used. *Oxygen*: Aerobic. *Temperature*: 22–35°C. *Habitat*: Soil.

Colony characteristics. *Starch*: Colonies that appear on starch agar in 3 or 4 days are smooth, glistening, and have an entire edge. A "rough" strain with somewhat irregular margin has also been isolated. In 5 or 6 days the colonies measure 2–3 mm. in diameter and may turn a pale yellow color. Subsurface colonies are disc-shaped. *Water-insoluble dextrin*: Very small colonies appear on water-insoluble cellulose dextrin in 5 or 6 days. In 9 days the colonies appear chalk-white, convex, and round with an entire edge. Submerged colonies are disc-shaped. Very distinct halos extend 2–5 mm. from the edge of the colonies. The latter usually measure 2 mm. in diameter.

Bacillus aporrhoeus n. sp.

Aporrhoeus is from the Greek, *aporrhoe*, meaning flowing outwards.

Morphology. *Vegetative cells*: Slightly curved rods with rounded ends, 3.4–4.5 x 0.8–0.9 microns, arranged singly. *Spores*: Present. Ellipsoid endospores are 1.7–2.0 x 0.9–1.1 microns, terminal to sub-terminal. Sporangia generally swollen terminally. *Motility*: Present, by peritrichous flagella. *Staining*: Gram positive.

Cultural characteristics. *Gelatin stab*: No growth or liquefaction. *Starch agar slant*: Heavy, slimy, white to transparent growth. *Litmus milk*: No visible growth. *Indole*: Negative. *Nitrate*: Nitrates are reduced to nitrites. *Diastase*: Positive. *Carbohydrates*: Glucose, maltose, galactose, arabinose, xylose are utilized. Lactose is not attacked. *Polysaccharides*: Cellulose, cellulosan, water-insoluble and water-soluble cellulose dextrans, hemicellulose, and pectin are utilized. *Filter paper strips*: Cellulose below the surface of the liquid becomes very limp, pulpy, and slightly swollen after about 7 days of incubation. The paper strips are broken in two only after slight shaking. The most extensive attack occurs at the surface of the liquid, where a pale yellowing is sometimes noticeable. The solution is never more than slightly turbid. *Nitrogen*: Peptone, yeast, nitrate, and ammonia are used. *Oxygen*: Aerobic. *Temperature*: Optimum 22–35°C. *Habitat*: Soil.

Colony characteristics. *Starch*: Colonies appear in 3 or 4 days. They are irregularly raised, gray-white in color, and semi-transparent. Growth is viscid and very gummy in consistency. Colonies vary from 2 to 6 mm. in diameter. Colonies often extend themselves by moving about on the surface of the agar medium and piling up, giving a windrow effect. A portion of the colony may move in a thin line away from the main body at the rate of 3 mm. in 2 hours.

Not all portions of the same colony or neighboring colonies move as vigorously as this, however. The movement may be such that a hooked or whorled effect is produced at the margin. Movement seems to occur more frequently on glucose agar than on starch. Not all the colonies move. *Water-insoluble dextrin*: Small, pin-point colonies are surrounded by a pronounced enzymatic zone and appear in 5 or 6 days. Ten-day-old colonies appear 1 to 2 mm. in diameter, convex in elevation, cloudy-white in color, and often irregular in shape. Growth is slow, but the enzymatic zone is pronounced, generally extending 2 to 3 mm. from the edge of the colony. Colony movement is infrequent.

DISCUSSION OF ORGANISMS

The taxonomy of the cellulose-decomposing bacteria has been confused by the policy of creating for these organisms special genera within the families to which they belong on morphological grounds. A physiological property has no place in a genus description unless the characteristic in question is obligate or so outstanding as to outweigh most other considerations. Almost all of the cellulose bacteria are versatile organisms, capable of utilizing other polysaccharides and carbohydrates to various degrees. In most cases the cellulose-decomposing ability does not outweigh the other characteristics. This property, therefore, is best relegated to the key, where it may well be conveniently used for separating species within the genus as morphologically described. The only exception to this might be the case of specific cellulose organisms. A few species which develop poorly on substrates other than cellulose are known but until their physiology has been more fully studied, it is probably unwise even to set these apart from other organisms closely related morphologically.

In view of the considerations above, the five new species described in this paper have been assigned to existing genera on morphological grounds. Four out of the five are versatile organisms; one has only been cultured on cellulose and cellulose-dextrin.

Three have been placed in the genus *Pseudomonas*. Some curved cells were usually present in cultures of each of these three species, but the proportion of curved cells did not justify inclusion in the genus *Vibrio*. Pigment production occurred to some extent in each of the three cultures; *P. ephemerozyanea*, as the name implies, produces an intense blue to blue violet color which is quite short-lived. When developing on filter paper strips, the coloration is somewhat localized to the area of attack at and immediately above the surface of the liquid. In aerated cellulose suspensions, the whole medium becomes blue. The pigment is not confined to the fibers and appears to be water-soluble. It is transitory, however, and after only a few hours fades slowly to a tan or light brown color. The ephemeral blue pigment has not been observed to be produced from any substrate other than cellulose but colonies on other carbohydrates on plates become light brown with age. A reddish pigment, also water-soluble, is produced by *P. erythra* on cellulose. The color is not intense and is most readily seen in filter paper strip cultures. By capillary action the pigment is carried up the filter paper until the whole portion exposed has a reddish hue. The area of

most vigorous attack may be pale brown. Colonies on cellulose dextrin agar are buff to red brown, but the color is limited to the colony itself. Pigment production is less evident in the third species of this genus, *P. lasia*. On filter paper strips the area attacked slowly becomes pale yellowish and colonies on cellulose dextrin and other substrates also assume this color as they age. It can hardly be claimed, however, that there is clear evidence of the formation of a water-soluble pigment in this case.

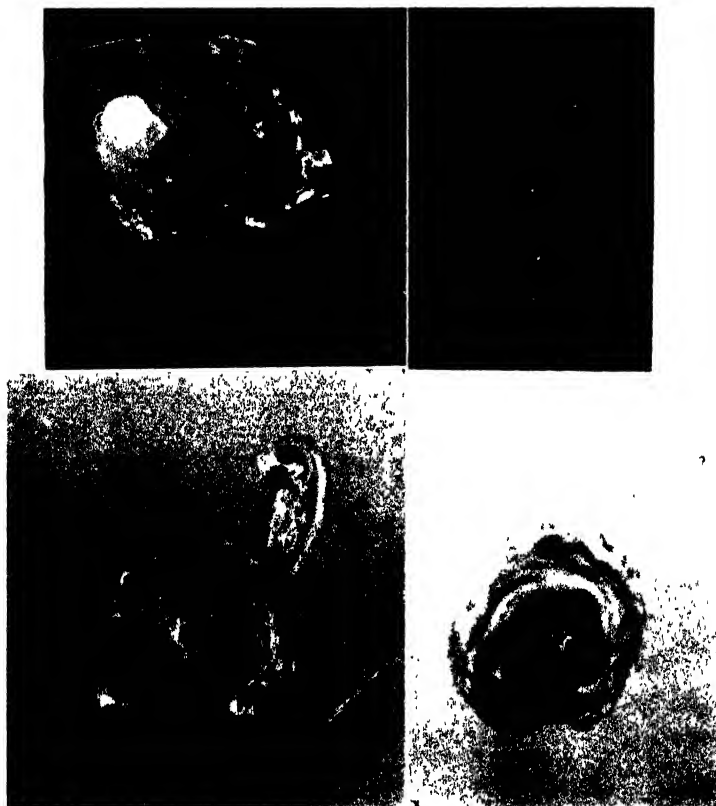


FIG. 1. MOTILE COLONIES OF *BACILLUS APORRHOEUS*

Pseudomonas erythra, unlike the other two species of this genus here described, seems limited to the utilization of cellulose. Only feeble growth is supported by cellulose dextrin, and no growth is obtained on starch or simple sugars. This organism is considerably less vigorous in its development on filter paper than the specialized cytophagae, several strains of which were isolated and studied in parallel with these new cultures.

Two strains of *Achromobacter picrum* were isolated, differing only slightly in colony appearance. In its fully aerobic character, this organism differs from most species of *Achromobacter*, the great majority of which are facultative. This organism is unusual in producing acids from cellulose aerobically. Acids are similarly given from many other carbohydrate substrates.

Aerobic spore-forming rods are rarely seen in soil unless some recent addition of plant materials has occurred. Even so, very few bacilli have been found to have the property of utilizing cellulose. One species, *Bacillus latvianus*, was described by Kalnins (1930), and two others, *Bacillus (Cellulobacillus) myxogenes* and *Bacillus (Cellulobacillus) mucosus* were studied in detail by Simola (1931). *Bacillus polymyxa*, *B. macerans* and *B. amylolyticus* have been reported to have feeble cellulose-decomposing powers though they are not ordinarily regarded as cellulose bacteria. Although its growth on filter paper is not as vigorous as some organisms, the cellulose-decomposing ability of *B. aporrhoeus*, here described, is unquestionable. It has in addition the extremely interesting and unusual property of giving motile or "amoeboid" colonies (fig. 1). Movement is frequently rapid but does not seem to occur on all media. Three other organisms of this genus, *B. circulans*, *B. sphaericus*, and *B. alvei* have been observed to behave similarly, but none of these utilizes cellulose. Inasmuch as colony motility is so distinctive a property this was made use of in arriving at the species name.

SUMMARY

Five new species of aerobic cellulose-decomposing bacteria are described. Three are species of *Pseudomonas* (*P. cphemerocyanea*, *P. lasia*, and *P. erythra*), one of *Achromobacter* (*A. picrum*), and one of *Bacillus* (*B. aporrhoeus*). With the exception of *P. erythra* all are versatile organisms capable of growing well on many carbohydrates. *A. picrum* alone produces acid from cellulose and sugars. *B. aporrhoeus* gives motile colonies on starch and glucose-agar.

ACKNOWLEDGMENT

We are much indebted to Dr. R. E. Buchanan for assistance in the nomenclature of these organisms.

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CELLULOSE DECOMPOSITION BY AEROBIC MESOPHILIC BACTERIA FROM SOIL

II. BIOCHEMICAL STUDIES ON FILTER PAPER AND CELLULOSE PREPARATIONS¹

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The conventional criterion employed in testing the ability of an aerobic organism to utilize cellulose is growth on filter paper. As ordinarily carried out, this test provides little information as to the activity of the culture. Moreover, there are grounds for believing that it may be too exclusive a criterion, because filter paper is hardly representative of cellulose as it occurs in the plant. The structural cellulosic framework of most plant tissues is not solely built up from the glucose polysaccharide, typified by cotton, that the chemist recognizes as pure cellulose. Other polysaccharides are normally present in the most intimate association with the "true" cellulose. These associated polysaccharides, conveniently termed cellulans, are not impurities, but form an integral part of the cellulosic fabric through which they are distributed. In chain length they are considerably shorter and perhaps more variable than the true cellulose component. The cellulans molecules are oriented and retained by lateral forces similar to those operative between cellulose chains. They are, however, removable by extraction with alkali, or by hydrolysis with dilute acids. Complete removal is a matter of great difficulty. In most plant celluloses the cellulans fraction is predominantly, but not exclusively, xylan. The effect of the presence of these cellulans on the availability of the cellulosic fabric containing them has not hitherto been investigated. Quantitative information was therefore sought as to the comparative activity of a number of aerobic cellulose bacteria on filter-paper and on cornstalk cellulose preparations, high and low in cellulans.

EXPERIMENTAL

Cellulose preparations

(a) *Cornstalk cellulose.* The cellulose was isolated from finely ground cornstalks by a modification of the procedure described by Norman and Jenkins (1933). All hypochlorite treatments were acidified. The cellulose was finally washed free from sulphite by repeated suspension in water and filtration, and was then dried at 60°.

(b) *Extracted cornstalk cellulose low in cellulans.* Prior to the isolation of cellulose by the method just mentioned, the cornstalks were extracted for one hour in flowing steam with an excess of one per cent NaOH. The cellulose prepared from this residue was further extracted by treatment, first with four per

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cent NaOH in flowing steam for three hours, and after washing, by treatment with five per cent H_2SO_4 for $3\frac{1}{2}$ hours. The residue was again subjected to extraction with 4 per cent NaOH for three hours under the same conditions. Even after these drastic treatments, about 25 per cent of the xylan remained with the cellulose.

(c) *Extracted cornstalk cellulose intermediate in cellulosan.* Partial removal of cellulosan was accomplished by heating cornstalk cellulose first with four per cent NaOH for three hours in flowing steam and then with five per cent H_2SO_4 for the same period.

Technique used in decomposition studies

Quantitative studies of the availability of the various cellulosic preparations were carried out in 1500 ml. round-bottom pyrex flasks containing 3 g. samples

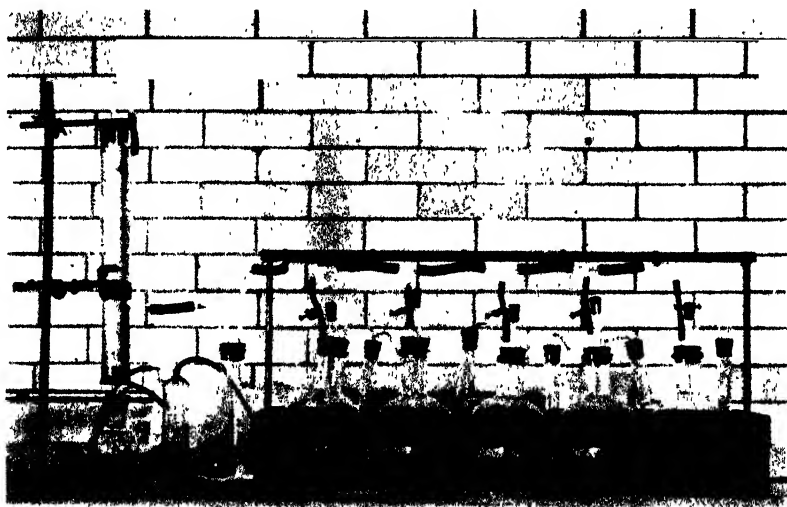


FIG. 1. EQUIPMENT USED IN CELLULOSE DECOMPOSITION STUDIES

of the finely divided cellulosic preparations suspended in 400 ml. nutrient solution. For convenience in sterilization, five flasks were mounted in a wooden crate (figure 1). Each flask was provided with an air inlet, inoculation tube, and air outlet with trap. Inoculation was effected by adding a suspension of the desired culture through the inoculation tube. The surface growth on a starch agar slant was used for each flask, if the organisms grew on starch. In the case of those organisms developing only on cellulose, a filter paper strip culture was employed, and the cellulose immediately above the sample of the liquid macerated to give a heavy suspension. After inoculation, a vigorous stream of sterile, moist, air was bubbled through the flasks. Apart from being necessary for the growth of the organisms, the air stream prevented the cellulose from settling out completely. Even so, occasional shaking was desirable.

At the close of the decomposition period, the air stream was stopped and 1 ml. of the supernatant, obtained when any residual cellulose had settled out, was

withdrawn. After appropriate dilution, this was plated on starch agar and cellulose dextrin agar as a test of purity of the contents of the flask. Contamination rarely occurred. The residual cellulose was recovered by centrifuging and thorough washing with water. In order to remove bacterial gum, the residual cellulose was treated for five minutes with 150 ml. of cold 0.1 per cent sodium carbonate, washed several times with water, followed by one per cent acetic acid, and water again. After transferring to a Gooch crucible, the excess water was removed by treatment with several portions of 95 per cent ethanol, before drying at 98° to constant weight. The alcohol treatment in some cases also removed alcohol-soluble pigments.

Determination of xylan

The furfural yield of the preparations and the residues therefrom was determined by the A.O.A.C. method. Sintered glass crucibles (1G4 Jena or F Pyrex) were used to filter the phloroglucide precipitates, which, after drying, were extracted with hot ethanol until colorless extracts were obtained. The furfural yields and xylan equivalents were taken from Krober's tables.

Organisms employed

Ten cultures were used in the decomposition studies. These included the five new species described in the previous paper (Fuller and Norman, 1943), one, *Achromobacter picrum* being represented by two strains, and four strains of *Sporocytophaga myxococcoides* (Hutchinson and Clayton emend. Krzemieniewska) Stanier. One of the latter (CG) was supplied by Dr. P. H. H. Gray of MacDonald College, Quebec, and the remainder were isolated by us from local soils. These organisms fall into two physiological groups. The cytophagas and *Pseudomonas erythra* are limited to the utilization of cellulose and some of the hydrolytic products of cellulose. The remainder are versatile organisms capable of developing on a wide range of substrates.

Decomposition of filter paper

The extent of the attack on filter paper cellulose accomplished by each of these cultures in 14 days is given in table 1. The separate trials with any one organism were not made concurrently. The more active organisms utilized in the neighborhood of one-third of the cellulose. One of the strains of *S. myxococcoides* was notably less active than the other three. Acid was produced only by the two strains of *Achromobacter picrum*. Inasmuch as the medium was poorly buffered and contained no carbonate, it is possible that the performance of these two strains was affected by the progressively increasing acidity. The agreement between the results obtained in separate trials was on the whole satisfactory; the less active cultures seemed somewhat more variable than the more vigorous ones.

Decomposition of cornstalk cellulose preparations

Each of the organisms accomplished more extensive decomposition of cornstalk cellulose than of filter paper cellulose in equal time. Three out of the four cytophagas were particularly vigorous and indeed in one trial all the cellulose

was utilized. *Pseudomonas ephemerocyanea*, which was the equal of these three cytophagas on filter paper, fell somewhat behind them on cornstalk cellulose. Those cultures which on filter paper were relatively inactive all brought about substantial decomposition of the cornstalk cellulose. These data indicate clearly that the potentialities of an organism in cellulose decomposition cannot

TABLE 1
Decomposition of filter paper cellulose in 14 days

ORGANISM	STRAIN	TRIAL	DECOMPOSITION	FINAL pH
			<i>per cent</i>	
<i>Pseudomonas ephemerocyanea</i>		1	34.7	7.0
		2	34.5	7.2
		3	30.9	7.1
<i>Pseudomonas lasia</i>		1	19.7	8.0
		2	4.5	8.0
<i>Pseudomonas erythra</i>		1	15.6	7.2
		2	5.8	7.2
		3	4.6	7.1
<i>Achromobacter picrum</i>	C4	1	27.5	4.8
		2	22.1	5.0
<i>Achromobacter picrum</i>	C2-1	1	27.7	5.2
		2	35.4	5.0
<i>Bacillus aporrhoeus</i>		1	4.2	7.1
		2	3.8	7.1
		3	1.7	7.0
<i>Sporocytophaga myzococcoides</i>	CG	1	32.8	7.6
		2	30.9	7.6
		3	32.0	7.6
<i>Sporocytophaga myzococcoides</i>	VC	1	6.3	7.5
		2	12.8	7.6
		3	13.9	7.6
<i>Sporocytophaga myzococcoides</i>	C3	1	33.2	7.6
		2	42.5	7.6
<i>Sporocytophaga myzococcoides</i>	C5	1	33.0	7.6
		2	34.9	7.6

be confidently predicted from studies carried out on a purified substrate, such as filter paper.

The cornstalk cellulose preparation contained 28.2 per cent xylan, the behaviour of which in decomposition is of great interest. The remainder, which was hexosan in nature, cannot be assumed to consist exclusively of long chain cellulose molecules though it would be predominantly of this nature. A certain

amount of short chain hexosan ordinarily occurs in plant celluloses, but this fraction cannot be distinguished by any routine analytical procedure. In table

TABLE 2
Decomposition of cornstalk cellulose in 14 days (xylan content 28.2 per cent)

ORGANISM	TRIAL	DECOMPOSITION	LOSS FROM 100 G. CELLULOSE		PER CENT REMOVAL	
			Xylan	Xylan-free cellulose	Xylan	Xylan-free cellulose
		<i>per cent</i>				
<i>P. ephemerocyanea</i>	1	73.9	25.7	48.2	91.1	67.1
	2	65.9	24.2	41.7	85.8	58.1
	3	47.4	22.3	24.1	79.1	33.6
<i>P. lasia</i>	1	37.4	22.5	14.9	79.8	20.8
	2	31.9	21.1	10.8	74.8	15.0
	3	36.0	21.9	14.7	77.7	19.6
<i>P. erythra</i>	1	54.9	22.3	32.6	79.1	45.4
	2	31.2	15.7	15.5	55.7	21.6
	3	42.4	16.6	25.8	58.9	35.9
<i>A. picrum</i> , strain C4	1	48.2	22.3	25.9	79.1	36.1
	2	30.2	18.1	12.2	64.2	17.0
	3	34.0	19.4	15.2	68.8	21.1
<i>A. picrum</i> , strain C2-1	1	43.1	22.0	21.1	78.0	29.4
	2	26.1	20.6	5.5	73.0	7.7
	3	28.3	20.2	8.1	71.6	11.3
<i>B. aporrhoeus</i>	1	48.9	22.8	26.1	80.9	36.3
	2	36.1	22.8	23.3	80.9	32.5
	3	30.4	19.7	13.7	69.9	19.9
<i>S. myzococcoides</i> , strain CG	1	93.3	27.4	65.9	97.1	91.8
	2	42.6	18.9	23.7	67.1	33.0
	3	91.5	26.7	64.8	94.7	90.3
<i>S. myzococcoides</i> , strain BC	1	59.8	20.9	38.9	74.1	54.2
	2	44.0	18.3	25.7	64.9	35.7
	3	46.0	18.4	27.6	65.2	38.4
<i>S. myzococcoides</i> , strain C3	1	81.0	25.3	55.7	89.7	77.6
	2	89.5	26.4	61.3	93.6	85.4
	3	81.1	26.2	61.9	92.9	86.2
<i>S. myzococcoides</i> , strain C5	1	68.7	23.1	45.6	81.9	63.5
	2	99.9	28.2*	71.7*	100.0	100.0
	3	83.3	25.6	57.7	90.8	80.4

* No residue for analysis.

2, therefore, the cornstalk cellulose is accounted for under the two headings of xylan and xylan-free cellulose.

The xylan fraction was utilized by all organisms including the specialized forms. In every case a greater per cent of it was removed than of the xylan-free cellulose fraction, and in many cases 30–40 per cent more xylan than xylan-free cellulose was lost. Since the xylan was attacked to a greater extent proportionately than the hexosan material through which it is distributed, preferential utilization of

TABLE 3
Decomposition of extracted cornstalk cellulose in 14 days (xylan content 7.2 per cent)

ORGANISM	TRIAL	DECOMPOSITION per cent	LOSS FROM 100 G. CELLULOSE		PER CENT REMOVAL	
			Xylan	Xylan-free cellulose	Xylan	Xylan-free cellulose
<i>P. ephemerocyanea</i>	1	44.0	2.3	41.7	31.9	44.9
	2	26.9	1.1	25.8	15.3	27.8
	3	51.5	4.3	47.2	59.7	50.9
<i>P. lasia</i>	1	3.9	0.3	3.6	4.2	3.9
	2	6.5	0.3	6.2	4.2	6.7
<i>P. erythra</i>	1	7.5	0.5	7.0	6.9	7.5
	2	7.0	0.5	6.5	6.9	7.0
<i>A. picrum</i> , strain C4	1	6.5	0.0	6.5	0.0	7.0
	2	9.0	0.2	8.8	2.8	9.5
	3	7.5	0.3	7.2	4.2	7.8
<i>A. picrum</i> , strain C2-1	1	5.3	0.3	5.0	4.2	5.4
	2	7.5	0.0	7.5	0.0	8.1
<i>B. aporrhoeus</i>	1	1.8	0.0	1.8	0.0	1.0
	2	0.2	0.0	0.2	0.0	0.2
<i>S. myzococcoides</i> , strain CG	1	67.0	4.7	62.3	65.3	67.1
	2	20.1	1.2	18.9	16.7	20.4
	3	47.2	3.4	43.8	47.2	47.1
<i>S. myzococcoides</i> , strain C3	1	18.7	1.2	17.5	16.7	18.9
	2	33.5	2.3	31.2	31.9	33.6
	3	55.5	3.9	51.6	54.2	55.6
<i>S. myzococcoides</i> , strain C5	1	43.5	3.4	40.1	47.2	43.2
	2	40.5	2.9	37.3	40.3	40.5

xylan must have occurred. This seems to have been particularly the case with those organisms which were not vigorous, such as *Achromobacter picrum* and *Pseudomonas lasia*. Approximately two-thirds of the material removed by these organisms was xylan. Less variation seemed to occur in the extent of attack on the xylan than on the hexosan fraction. For example, the differences between the three trials with *P. ephemerocyanea* were due primarily to differences in the extent of decomposition of the xylan-free cellulose.

Although from 75–80 per cent of the xylan could be removed without a proportionate attack on the hexosan fraction, the remainder seemed less available and to be utilized only as the hexosan fraction was extensively decomposed.

Additional information as to the effect of the presence of xylan is obtained by comparison with the results in table 3. Cornstalk cellulose, drastically treated with acid and alkali in order to remove much of the xylan, was far less extensively attacked than the untreated cellulose, and resembled filter paper in behaviour. Only the three strains of *S. myzococcoides* and *P. ephemerocyanea* accomplished appreciable decomposition. There was no indication that the residual 7.2 per cent xylan was available to be used preferentially by these or the less vigorous organisms or that its presence exerted any influence on the availability of the

TABLE 4

Decomposition of extracted cornstalk cellulose in 14 days (xylan content 12.4 per cent)

ORGANISM	TRIAL	DECOMPOSITION
		<i>per cent</i>
<i>P. ephemerocyanea</i>	1	68.3
	2	42.3
<i>P. erythra</i>	1	65.5
	2	53.3
<i>A. picrum</i> , strain C4	1	28.9
<i>B. aporrhoeus</i>	1	64.1
	2	24.2
<i>S. myzococcoides</i> , strain CG	1	68.2
	2	54.6
<i>S. myzococcoides</i> , strain VC	1	53.9
	2	51.9
<i>S. myzococcoides</i> , strain C3	1	38.1
	2	44.2

cellulose. Indeed the utilization of the xylan was in all cases proportionate to the attack on the hexosan fraction.

A few trials were carried out on a sample of cornstalk cellulose less drastically extracted and containing 12.4 per cent xylan (table 4). This material was distinctly more available and more extensively utilized than the low xylan sample, particularly by the less vigorous organisms, such as *Pseudomonas erythra*, *Achromobacter picrum*, and *Bacillus aporrhoeus*. Separate xylan determinations were not carried out on the individual residues.

DISCUSSION

On the basis of the rate at which they accomplish the decomposition of filter paper, most aerobic cellulose-decomposing bacteria cannot be regarded as being

particularly vigorous. It is difficult to reconcile such observations with the rapidity of decomposition of the cellulosic fraction of plant materials in soil or compost heaps. The explanation of this apparent discrepancy can now be given. Plant celluloses normally contain 20–30 per cent of associated polysaccharides in the cellulosic fabric, and the presence of these cellulosans exerts a favorable influence on availability. The cellulosan component is itself more readily available than the long-chain “true” cellulose component, and may therefore be preferentially utilized by some organisms. Apart from this, however, the decomposition of the hexosan appears to be aided by the presence of the cellulosan. Cellulose is so insoluble and the production of exo-enzyme apparently so restricted that the organisms have to be in contact with the fibers for decomposition to occur. The greater availability of the cellulosan probably permits of the more rapid development of a large population.

In the cornstalk cellulose studied, not all the xylan was of equal availability. About three-fourths of that present was readily removed without a proportionate attack on the hexosan. The remaining fourth seemed to be utilized only concurrently with the cellulose with which it was associated. In the extracted cornstalk cellulose, drastically treated to remove xylan, about one-fourth the xylan remained, but this was not preferentially utilizable and its presence no longer exerted any influence on the decomposition of the material as a whole. These observations throw some light on the relationship between cellulosan and cellulose. The similarity in behaviour of the major part of the cellulosan to hydrolysis or extraction, and to enzymic hydrolysis by bacteria cannot be overlooked. That fraction which resisted drastic chemical treatment was also less available biologically. To account for the extreme resistance of a portion of the cellulosan to extraction with chemical agents, the theory has been advanced that there may be imperfect cellulose chains containing both glucose and xylose units (Norman, 1936). The presence of a few such mixed chains could account for the stability of that portion of the xylan which resists extraction. Moreover, it would be expected that such xylose groups could be utilized only at the same rate as the hexose groups if these chains were attacked. This was in accord with the results observed in the trials with extracted cornstalk cellulose.

SUMMARY

Quantitative studies were made of the utilization of filter paper and cornstalk cellulose preparations by ten cultures of aerobic cellulose-decomposing bacteria. Five new species were included, *Pseudomonas ephemerozyanea*, *Pseudomonas lasia*, *Pseudomonas erythra*, *Achromobacter picrum*, and *Bacillus apporhoeus*, together with four strains of *Sporocytophaga myxococcoides*.

The more active organisms utilized about one-third of the filter paper supplied in fourteen days. Cornstalk cellulose was far more extensively decomposed by all organisms in equal time. The presence of xylan in the cellulosan component of the cornstalk cellulose exerted a favorable influence on decomposition. The xylan was utilized extensively by all organisms including those forms believed to be specialized. About 75 per cent of the xylan was more readily available than the remaining fourth.

Extracted cornstalk cellulose, so treated to remove the major part of the xylan, resembled filter paper in availability. The xylan remaining was not preferentially attacked, and appeared to be no more available than the cellulose with which it was associated. Biological support is provided for the opinion that a portion of the xylan may be present in imperfect cellulose chains.

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CELLULOSE DECOMPOSITION BY AEROBIC MESOPHILIC BACTERIA FROM SOIL

III. THE EFFECT OF LIGNIN¹

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It is a matter of common observation that lignified plant materials are more slowly and less extensively decomposed than unlignified materials. Lignin, though not wholly unavailable, is utilized far less readily than the other cell-wall constituents, even under the most favorable circumstances. Plant tissues cannot be regarded as simple mixtures of organic compounds. The cell-wall fabric, though predominantly cellulosic, is a structure both infiltrated and incrustated with lignin and polyuronide hemicelluloses, the presence and distribution of which may reasonably be expected to influence the extent of attack on the cellulose. The effects may not be directly proportionate to the amounts of the other constituents present. In general, however, above a certain point, the extent to which plant materials decompose decreases as the lignin content increases. Such a material as coir fiber, with a lignin content of over 35 per cent, is quite resistant even though the major constituent is cellulose. Olson, Peterson, and Sherrard (1937) examined the effect of the presence of lignin in wood pulps and similar products on the fermentation of the cellulose by thermophilic enrichment cultures. In order to obtain extensive fermentation (85 per cent or more), the lignin content had to be less than 1 per cent. The presence of 2-4 per cent lignin in pulps reduced the amount of cellulose fermented to 50-60 per cent. The addition of isolated lignin, or lignin-containing materials such as ground wood, did not interfere with the fermentation of pure cellulose. They concluded that the effect produced by the presence of lignin is not physical but due to chemical union between lignin and cellulose.

In the experiments reported herein the availability of cellulose in a series of jute preparations delignified to different degrees was determined. Organisms, the behavior of which on filter paper and cornstalk cellulose had previously been determined (Fuller and Norman, 1943) were employed. Jute was selected as an experimental material because it consists almost solely of cellulose, lignin, and polyuronide hemicelluloses. The content of protein is extremely low.

EXPERIMENTAL

Preparation of jute samples. Raw jute fiber was finely ground and extracted thoroughly with hot water. After drying, a sample of jute cellulose was prepared by delignification with monoethanolamine at 170°C. for five hours, according to the procedure of Wise *et al.* (1939). The product, after washing with alcohol,

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was chlorinated in suspension in water, and extracted with boiling 3 per cent sodium sulphite in order to remove the last traces of lignin. Preparations delignified to different extents were obtained by giving monoethanolamine treatments for periods of $\frac{1}{2}$, 3 and 7 hours without subsequent chlorination.

Decomposition technique. The procedure used in the decomposition studies was as described by Fuller and Norman (1943). Three trials were carried out on each preparation but not concurrently. Each experiment consisted of one sample from each of the five preparations inoculated uniformly from equal-age cultures of one organism.

Organisms employed. On the basis of the experiments carried out on filter paper and cornstalk cellulose, four cultures were selected for use. These were *Pseudomonas ephemerocyanea*, *Achromobacter picrum*, *Bacillus aporrhoeus*, and *Sporocytophaga myxococcoides*. Four strains of the last were tested in previous work, and the one which was consistently vigorous was employed. This was strain CG, originally obtained from Dr. P. H. H. Gray of Macdonald College.

TABLE 1
Analyses of jute preparations
Results expressed as g. per 100 g. sample

PREP. NO.	CELLULOSE	LIGNIN	FURFURAL FROM CELLULOSE	XYLAN IN CELLULOSE	TOTAL FUR- FURAL YIELD	FURFURAL NOT FROM CELLULOSE
1	99.2	0.0	9.68	15.0	10.16	0.5
3	95.5	3.3	9.53	14.8	10.15	0.6
4	89.2	6.3	9.10	14.1	10.37	1.3
5	82.7	11.9	7.72	12.0	10.05	2.3
6	75.6	12.6	7.50	11.6	10.48	3.0

Decomposition of jute preparations. The effect of the ethanolamine treatments in accomplishing lignin removal may be seen from table 1. The polyuronide hemicelluloses, of which no direct measure can be obtained, are also extracted by this reagent. The yield of furfural, not from cellulose, may be taken as an indication of the amount of this constituent present in each sample. Sample 6 was the untreated jute fiber and sample 1, the jute cellulose, which contained about 15 per cent xylan. It will be noted that concurrently with the removal of lignin, polyuronide hemicelluloses were also extracted.

The results of the decomposition studies are presented in tables 2-5. The cellulose removed of course accounts either for all or for the major part of the loss suffered by the preparations if decomposition was extensive. It should be pointed out that the figure for cellulose removed includes the xylan present as cellulosan. In the last two columns, however, the per cent decomposition of the hexosan and pentosan components of the cellulose are given separately. These are calculated on the amounts of each present in the preparation (see table 1) and not on 100 g. of the sample. The purpose of this is that a basis of comparison is provided with the studies on cornstalk cellulose reported previously (Fuller

TABLE 2

Decomposition of jute preparations by Pseudomonas ephemerocyanea in 21 days

PREP. NO.	TRIAL NO.	TOTAL LOSS	LOSS FROM 100 G. SAMPLE			DECOMPOSITION PER CENT	
			Cellulose	Xylan in cellulose	Furfural not from cellulose	Xylan free cellulose	Xylan in cellulose
		<i>per cent</i>	<i>g.</i>	<i>g.</i>	<i>g.</i>		
1	1	86.9	86.9	13.5		87.2	89.9
	2	99.7	99.7	15.0		100.0	99.9
	3	100.0	100.0	15.0		100.0	100.0
3	1	73.2	71.6	12.5	0.5	73.2	84.5
	2	95.4	91.6	14.4	0.0	95.6	97.4
	3	59.0	58.1	11.2	0.5	58.1	75.7
4	1	54.4	54.3	10.6	1.0	58.2	75.0
	2	80.3	75.1	12.7	1.0	83.1	89.9
	3	45.8	43.7	10.5	1.1	44.2	74.4
5	1	21.3	18.8	2.9	2.0	22.5	24.2
	2	22.6	19.2	6.6	1.5	17.8	55.1
	3	38.3	35.9	9.1	1.3	37.9	76.0
6	1	14.7	7.6	1.4	1.4	9.7	12.0
	2	17.8	10.7	1.5	1.4	14.4	12.9
	3	18.6	13.7	2.4	0.8	17.7	20.6

TABLE 3

Decomposition of jute preparations by Sporocytophaga myzococcoides (strain CG) in 21 days

PREP. NO.	TRIAL NO.	TOTAL LOSS	LOSS FROM 100 G. SAMPLE			DECOMPOSITION PER CENT	
			Cellulose	Xylan in cellulose	Furfural not from cellulose	Xylan free cellulose	Xylan in cellulose
		<i>per cent</i>	<i>g.</i>	<i>g.</i>	<i>g.</i>		
1	1	52.1	51.3	7.5		52.0	50.0
	2	87.6	86.8	14.5		85.9	96.7
	3	76.4	75.6	12.9		74.7	86.0
3	1	65.0	62.6	10.5	0.1	61.8	71.0
	2	61.3	58.6	10.2	0.5	59.9	69.0
	3	78.1	75.4	12.2	0.3	78.3	82.5
4	1	34.6	27.7	7.0	1.0	27.5	49.6
	2	24.4	21.4	5.4	1.3	21.3	38.2
	3	46.9	42.3	7.9	0.6	45.8	55.9
5	1	11.7	6.6	3.2	1.1	4.8	26.7
	2	21.7	17.1	4.1	1.7	18.4	34.2
	3	22.7	17.6	3.7	1.4	19.6	30.9
6	1	4.7	1.9	0.0	1.2	3.0	0.0
	2	10.4	5.1	1.2	1.1	6.1	10.3
	3	5.3	3.4	0.0	0.8	5.3	0.0

TABLE 4

Decomposition of jute preparations by Archomobacter picrum (strain C4) in 21 days

PREP. NO.	TRIAL NO.	TOTAL LOSS <i>per cent</i>	LOSS FROM 100 G. SAMPLE			DECOMPOSITION PER CENT	
			Cellulose	Xylan in cellulose	Furfural not from cellulose	Xylan free cellulose	Xylan in cellulose
1	1	8.1	8.1	3.2		5.8	21.3
	2	10.2	10.2	4.1		7.3	27.3
	3	18.4	16.4	7.5		10.5	50.0
3	1	43.7	41.6	10.1	0.2	39.0	68.3
	2	34.2	32.2	9.4	0.6	28.2	63.6
	3	45.5	43.5	11.1	0.2	40.1	75.1
4	1	39.1	35.0	9.7	1.1	33.7	68.7
	2	30.2	28.1	10.7	1.0	23.2	75.8
	3	33.6	31.0	10.0	0.6	27.9	67.6
5	1	26.0	21.1	6.1	1.5	21.1	50.9
	2	35.1	30.7	7.6	1.5	32.7	63.4
	3	39.4	34.5	7.3	1.8	38.5	60.9
6	1	13.6	7.6	2.4	0.3	8.1	20.6
	2	12.9	6.3	2.1	0.8	6.6	19.9
	3	15.2	7.7	2.2	0.8	8.6	20.0

TABLE 5

Decomposition of jute preparations by Bacillus aporrhoeus in 21 days

PREP. NO.	TRIAL NO.	TOTAL LOSS <i>per cent</i>	LOSS FROM 100 G. SAMPLE			DECOMPOSITION PER CENT	
			Cellulose	Xylan in cellulose	Furfural not from cellulose	Xylan free cellulose	Xylan in cellulose
1	1	9.3	9.3	4.5		5.7	30.0
	2	14.0	14.0	5.8		9.7	28.6
3	1	11.0	10.8	6.8	0.3	5.0	46.0
	2	12.3	12.3	6.6	0.6	7.1	44.6
	3	10.3	9.4	5.4	0.6	5.0	36.5
4	1	12.8	11.5	7.2	1.0	5.8	51.0
	2	11.7	9.6	7.2	1.0	3.2	51.0
	3	9.5	7.0	7.0	0.1	0.0	49.6
5	1	12.8	11.1	6.1	1.8	13.4	50.9
	2	13.8	13.1	6.0	1.6	15.8	50.1
	3	13.2	10.7	5.9	1.6	12.9	49.2
6	1	5.4	2.4	0.8	0.8	3.2	6.9
	2	6.1	1.6	0.5	1.5	2.1	4.3
	3	9.8	4.2	1.4	0.4	5.6	12.0

and Norman, 1943), and so that preferential utilization of xylan, if it occurs, may be readily seen.

The utilization of cellulose by the four organisms was not equally affected by the presence of lignin. The measure of agreement between trials was not as good as when pure cellulose only was concerned. *P. ephemerocyanea* was the most vigorous organism and completely decomposed the jute cellulose in two out of three trials. Untreated jute fiber with 12.6 per cent lignin, on the other hand, was less than 20 per cent utilized, and almost half of the loss was due to removal of polyuronide hemicellulose, not cellulose. The extent of decomposition of the samples of intermediate lignin content increased as the lignin content declined. Upwards of 50 per cent decomposition occurred with the preparation containing 6.3 per cent lignin.

The results obtained with *S. myxococcoides* were somewhat similar, though at a lower level. Decomposition in excess of 50 per cent was accomplished only in trials with the preparation containing 3.3 per cent lignin, and with jute cellulose. The untreated jute fiber was only slightly attacked by this organism. The xylan in the cellulose of all preparations suffered greater loss proportionately than the hexosan component, though not to the extent of pronounced preferential utilization.

With *A. picrum* the extent of decomposition of the isolated jute cellulose was little, if any, greater than that of the untreated fiber, but the partially delignified samples were attacked to a considerably greater extent. This organism had previously been shown to utilize 30-40 per cent of cornstalk cellulose and 25 per cent of filter paper cellulose in a shorter period. Its restricted behavior on jute cellulose is, therefore, difficult to explain. The lignin content of the partially delignified samples did not seem to have much influence on the extent of their decomposition. In all cases, however, the xylan contributed disproportionately to the fraction removed.

The remaining organism, *B. aporrhoeus* was the least vigorous of the four, and, like *A. picrum*, found the isolated jute cellulose scarcely more available than the untreated jute fiber. Again, the lignin content did not appear to affect the limited amount of decomposition effected. The hexosan component of the cellulose suffered only small loss. Much of the material removed could be accounted for as xylan in the cellulose or as polyuronide hemicellulose.

DISCUSSION

The scope of these experiments is not great enough to allow of broad generalization. It is clear, however, that the presence of lignin substantially reduced the extent of the attack on the cellulose of the jute fiber that could be accomplished by the two vigorous cellulose-decomposing organisms. Whether the approach of partial delignification followed in this work is entirely satisfactory as a means of studying the effect of different lignin levels is not certain. A comparison of the behavior of preparation 5 which contained 11.9 per cent lignin with that of the untreated jute fiber containing 12.6 per cent lignin suggests that there may be an effect on availability caused by the treatment given in delignification but

separate from that due to the removal of lignin. The cellulose of preparation 5 was distinctly more available to all organisms than might have been expected, solely as a result of the removal of 0.7 per cent of lignin. The treatment concurrently removed about one-fourth of the polyuronide hemicelluloses, the loss of which, if it affected the extent of decomposition at all, would do so in the opposite direction.

The two less vigorous cellulose-decomposing organisms did not seem to be much affected by the amount of lignin present. Both utilized the xylan component of the cellulose to a disproportionate degree. It is not difficult to reconcile the fact that the presence of lignin appeared to exercise a greater effect on the vigorous organisms than on those which were not particularly effective in the utilization of jute cellulose if the composite structure of the lignified cell wall is borne in mind. The production of extra-cellular enzymes by cellulose bacteria seems to be restricted. No authentic reports of the hydrolysis or dissolution of cellulose by cell-free suspensions have been given. It has often been remarked that the organisms appear to have to be in contact with the fibers if extensive decomposition is to occur. Photographs of decomposing fibers show a heavy distribution of organisms on and through the fiber (for example, see Stanier, 1942). The distribution of lignin in lignified cell walls is such that the amount of cellulosic surface exposed to attack is reduced. Ball-mill grinding, as studied by Olson *et al.* (1937) would rupture the macro-cellular structure of a tissue or fiber, but still would not much change the micro-structure or alter the fact that the lignin and the cellulose are interpenetrating systems. It does not therefore seem necessary to conclude that lignin must exercise its effect in reducing the availability of cellulose because it exists in a chemical union with the cellulose, but simply that contact between organisms and cellulose surfaces with consequent production of extracellular enzymes is hindered. Our previous studies showed that unspecialized and specialized cellulose organisms vary considerably in ability to utilize pure unligified cellulose. The variation is more probably due to differences in the amount and activity of the extra-cellular cellulose systems produced than to varying abilities in the utilization of the soluble products produced thereby. It is likely that the extent of decomposition of the jute preparations produced by the less vigorous organisms was limited more by the inadequacy of the enzyme system to bring about rapid decomposition than by the presence of lignin. The more vigorous organisms, on the other hand, were prevented by lignin from full access to all the cellulose and, therefore, though capable of decomposing almost completely the isolated jute cellulose, the extent of attack was diminished at the higher lignin levels. Our explanation of the effect of lignin on cellulose availability is therefore primarily a physical one.

SUMMARY

The effects of the presence of lignin on the utilization of cellulose by four aerobic cellulose-decomposing bacteria has been determined. A series of preparations with decreasing lignin content was obtained from jute fiber by treatments with monoethanolamine.

The extent of decomposition accomplished by the vigorous organisms, *Pseudomonas ephemerocyanea* and *Sporocytophaga myxococcoides*, increased as the lignin content was reduced. Less vigorous organisms, such as *Achromobacter picrum* and *Bacillus apporhoeus* were little affected by the lignin content of the substrate. Both utilized the xylan component of the cellulose disproportionately.

Because lignin and cellulose in the cell-wall form interpenetrating systems, the effects of lignin in reducing the availability of cellulose, are probably mainly physical.

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PRODUCTION AND ACTIVITY OF STREPTOTHRICIN^{1,2}

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INTRODUCTION

The actinomycetes represent a widely distributed group of organisms which comprise many forms possessing marked antagonistic properties against bacteria, fungi and other microorganisms (Waksman, 1941; Waksman, Horning, Welsch and Woodruff, 1942; Welsch, 1942). The antibiotic substances produced by actinomycetes vary greatly in chemical nature, in specific antibacterial action, and in toxicity to animals. Actinomycin is by far the most toxic of these substances and streptothricin apparently the least. Because of this low toxicity, as well as its solubility in water, selective action against gram-negative and gram-positive bacteria (Waksman and Woodruff, 1942a; Waksman and Woodruff, 1942b), activity *in vivo* (Metzger, Waksman, Pugh, 1943; Robinson, 1943), relative stability and heat resistance, all of which make the antibacterial properties of streptothricin of particular importance, it was selected for further study.

Streptothricin is produced by an aerobic, conidia-forming species of actinomycetes which was identified, on the basis of its pigmentation and certain cultural characteristics, with an organism, isolated from the soil many years previously, as *Actinomyces lavendulae* (Waksman, Horning, Welsch and Woodruff, 1942; Waksman and Woodruff, 1942a). This organism is grown in simple media containing glucose or starch as a source of carbon, a protein digest (known under the trade name of tryptone) or different amino acids (glycine, glutamic acid) as a source of nitrogen, with limited concentrations of inorganic salts and tap water. The media are distributed in shallow layers in flasks, sterilized as usual, inoculated, and incubated at 28°C. for 7–12 days. The antibiotic substance is readily secreted into the metabolic solution. It is isolated from the culture filtrate by adsorption on active charcoal (norit-A), from which it is removed by treatment with acidified alcohol. The alcoholic extract is neutralized with NaOH solution and treated with 10 volumes of ethyl ether. This results in a highly concentrated

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² The author gratefully acknowledges the assistance obtained in this work from several collaborators. Dr. W. Kocholaty, of the University of Pennsylvania, first isolated in our laboratory the strain of the organism producing streptothricin and first tested it for its antagonistic action; Dr. E. Horning isolated in our laboratory several other species which produced streptothricin-like substances; Dr. H. B. Woodruff, now with Merck & Co., collaborated in the earlier work on the development of suitable media for the growth of the organism, as well as in the isolation and concentration of the streptothricin; Mr. A. Schatz, now with the U. S. Army, assisted in some of the experimental work reported in this paper. The author is also indebted to Merck & Co. for the concentrated and purified streptothricin used in some of these experiments.

aqueous solution of streptothricin. The active solution may be further concentrated by evaporation at reduced pressure.

Although the original culture of *A. lavendulae* isolated from the soil in 1915 is still available in the culture collection, some of its cultural characteristics have become changed to a considerable extent, so that the culture now differs from its original description. On careful testing, this culture was found to possess antagonistic properties, but only to a very limited extent; it was largely active against various gram-positive spore-forming bacteria.

Considerable difficulty in obtaining the active substance has been experienced by various investigators to whom cultures of streptothricin-producing strains of *A. lavendulae* have been submitted. Because of this and also because of the paucity of information concerning the best methods for culturing actinomycetes as well as of their physiological properties, the following investigations were undertaken. The primary purpose was to establish the optimum conditions for the production of streptothricin, the relation of this substance to the growth and physiology of the organism, strain variation and its formation by related organisms, and the mechanism of its production.³

EXPERIMENTAL

Methods

Actinomyces lavendulae is capable of producing streptothricin only when grown in very shallow layers in stationary cultures, the presence of abundant oxygen being essential to this process. A culture medium consisting of 10 grams anhydrous glucose (or starch), 5 gm. protein digest, 0.5 gm. K_2HPO_4 , 0.5 gm. NaCl, and 0.1 gm. $FeSO_4$, and 1000 ml. tap water, is inoculated with the lavender to rose-lavender colored spores of the organism grown on synthetic (glucose-asparagine) agar for 5–10 days, at 28°. When the inoculated spores drop to the bottom of this medium, they germinate and grow there into colonies, but do not give rise to a surface pellicle or produce only a ring on the surface along the glass; under these conditions only a little streptothricin is produced. The addition of 0.25 per cent agar to the medium was found to overcome this difficulty; however, the presence of agar makes the process of filtration of the cultures and the extraction of the active material rather cumbersome. In most of this work, 250 ml. portions of medium were placed in 1 liter Erlenmeyer flasks and were sterilized either in flowing steam, when glucose was used, or under pressure with starch as the carbohydrate source.

The ability of actinomycetes to grow in a submerged condition and to form streptothricin, provided the cultures are aerated by agitation, has recently been demonstrated by Woodruff and Foster (1943). A comparison has, therefore, been made of the production of streptothricin in stationary and in shaken cultures.

The antibiotic activity of streptothricin was measured by the agar plate-

³ The results presented in this paper and those of Woodruff and Foster presented elsewhere (1943) may be considered as supplementing one another, since they were carried out with the same organism, but in different laboratories.

dilution method, using several test organisms (Waksman and Woodruff, 1942b). The results are reported in terms of dilution units, as expressed by the ratio of the volume of test medium used (10 ml. agar) to the amount of culture required to give complete inhibition of the test organism. If partial inhibition was obtained in one dilution and complete inhibition in the next lower dilution, the end point was interpolated between these two dilutions. It has been pointed out elsewhere (Waksman and Horning, 1943) that this method has certain advantages and disadvantages, when compared with the dilution culture and agar-cup methods. When it is desired to employ several test organisms, especially when the active substance has not yet been isolated in a pure state, and when accurate quantitative yields are of only secondary consideration, this method is far superior to any other now in use for the study of antibiotic substances.

The four test organisms commonly employed in this laboratory for the study of antibiotic substances are *Escherichia coli*, *Bacillus subtilis*, *Bacillus mycoides* and *Sarcina lutea*. Streptothricin has very little activity against the third and its action against the fourth is similar to that upon *E. coli*. Hence, only the results obtained against the first two organisms are reported here. It is of particular interest to note that the ratio of activity of streptothricin against *E. coli* and *B. subtilis* is about 1:5 to 1:10. Although in most cases the results are presented in terms of both units, only one of these can readily be used as the test organism for measuring the activity of streptothricin. Since different strains obtained from different laboratories may vary in their sensitiveness, only well-defined test strains are to be employed.

Strain specificity

Several cultures of actinomycetes isolated from soil and from dust were found capable of producing streptothricin or a streptothricin-like substance (Waksman, Horning, Welsch and Woodruff, 1942). The culture of *A. lavendulae* largely used in this work was found to be made up of different strains which varied in their capacity to produce the antibiotic agent, as shown in table 1. These strains were isolated from the mother culture by plating suspensions of spores from agar slants and picking individual colonies. Some of the strains thus isolated, notably Nos. 3, 8, 12 and 14, gave fairly high antibacterial activity, whereas other strains, such as Nos. 4, 5, 6 and 10, produced no activity at all or only very little when grown in the same medium and under the same conditions of culture. Because of the great variation in the growth of the organism in stationary cultures and the resulting yields of streptothricin, it is not possible to state definitely that these variations are of a permanent nature. Considerably less variation was obtained in shaken cultures.

Two of the above strains, Nos. 8 and 14, were used chiefly in the experiments reported here. These two strains also varied from one another, as brought out in table 2. In shaken cultures, No. 8 gave greater activity, both in the tryptone and in the glycine media, than did No. 14; in stationary cultures the reverse was true.

TABLE 1

The production of streptothricin by different strains of A. lavendulae
Glucose-tryptone-soft agar medium

STRAIN NUMBER	E. COLI	B. SUBTILIS
	units	units
1	5	30
2	20	200
3	50	300
4	0	0
5	0	0
6	0	5
7	5	20
8	75	>300
9	5	50
10	3	20
11	20	100
12	75	300
13	25	150
14	75	>300
15	15	100
16	15	100

TABLE 2

Comparative activity of two strains of A. lavendulae
1 per cent starch media

STRAIN NUMBER	SOURCE OF NITROGEN	TREATMENT OF CULTURES	INCUBATION	GROWTH	ACTIVITY	
					E. coli	B. subtilis
			days	mg. per 100 ml. medium	units	units
8	Tryptone	Shaken	2	346	150	1,000
14	Tryptone	Shaken	2	361	150	750
8	Tryptone	Shaken	5	253	100	1,000
14	Tryptone	Shaken	5	296	100	500
8	Glycine	Shaken	2	162	30	30
14	Glycine	Shaken	2	146	30	30
8	Glycine	Shaken	5	266	100	500
14	Glycine	Shaken	5	271	30	150
8	Tryptone	Stationary	8	245	20	200
14	Tryptone	Stationary	8		75	300
8	Glycine	Stationary	8	239	25	150
14	Glycine	Stationary	8		75	200

Influence of nutrition of the organism upon the production of streptothricin

In view of the importance of the carbon and nitrogen sources in the growth of *A. lavendulae* and in the production of the streptothricin, the results of several typical experiments are reported here.

Four different forms of nitrogen, in concentrations of 0.3-0.5 per cent, with

starch (1 per cent) as the added source of carbon were compared, using stationary cultures. The maximum production of streptothricin was obtained in seven

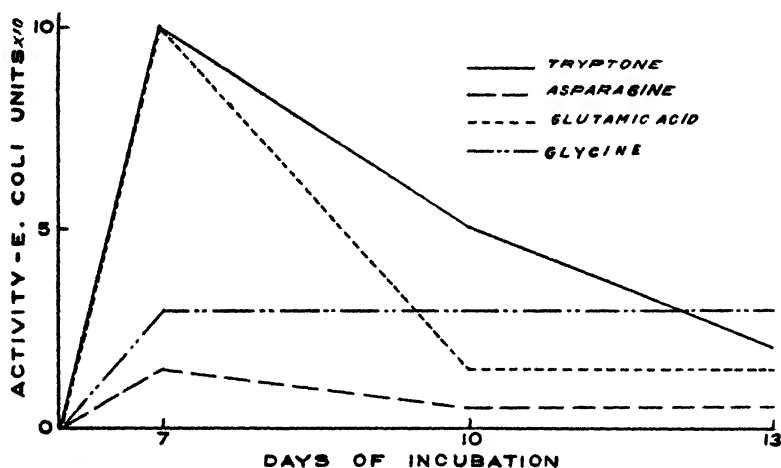


FIG. 1. INFLUENCE OF DIFFERENT FORMS OF NITROGEN UPON THE PRODUCTION OF STREPTOTHRICIN—*E. COLI* UNITS

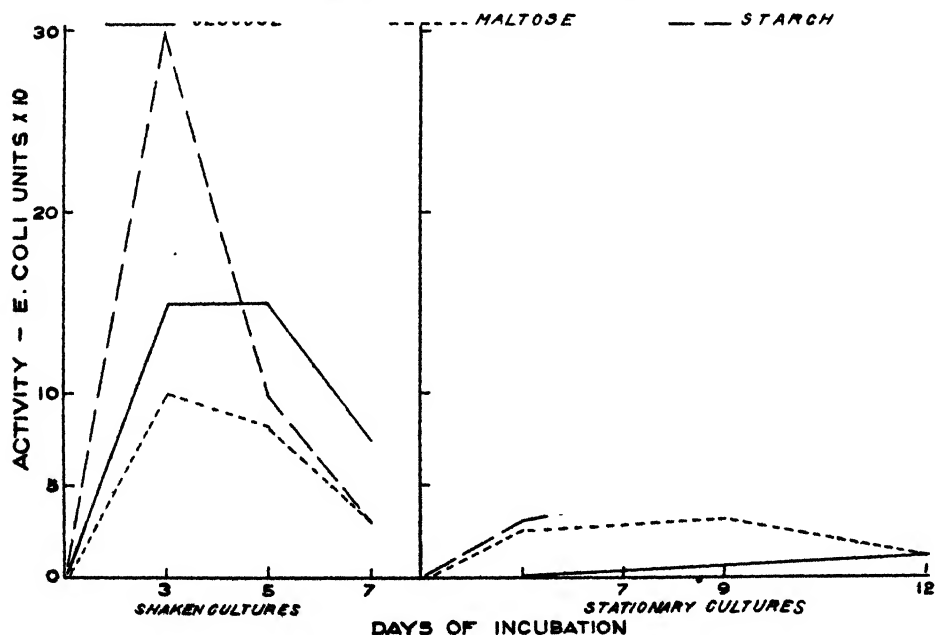


FIG. 2. INFLUENCE OF DIFFERENT CARBON SOURCES UPON THE PRODUCTION OF STREPTOTHRICIN

days, followed by a rapid decrease. The protein digest tryptone and glutamic acid proved to be the best sources of nitrogen; however, growth was not uniform in all the flasks, especially in the glutamic acid cultures, some showing little if any

activity. The activity with glycine was not so high but tended to remain constant (fig. 1). Shaken cultures were found to give similar results, with a slight difference, tryptone giving the highest activity in 3 days (200 *E. coli* units) and glycine in five days (150 units); asparagine gave the lowest activity in shaken cultures as well.

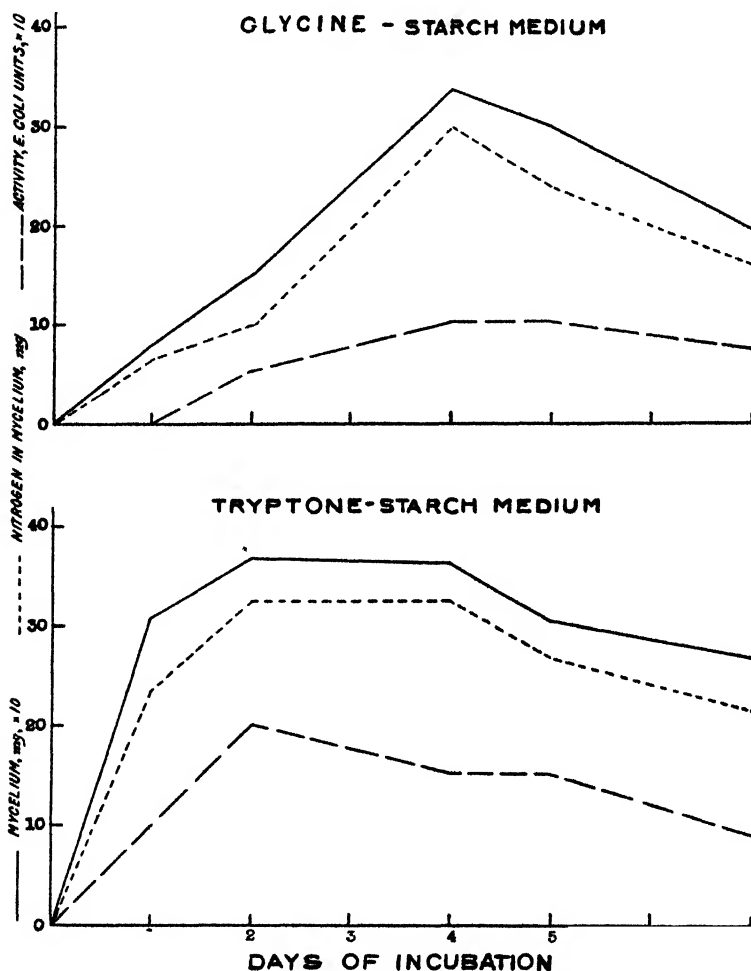


FIG. 3. INFLUENCE OF NITROGEN SOURCES UPON THE GROWTH OF *A. LAVENDULAE* AND FORMATION OF STREPTOTHRICIN

A comparative study of the effect of different carbon sources upon the production of streptothricin gave the highest activity for starch, followed by maltose or by glucose. Starch had the added advantage that one could do away with the use of agar in stationary cultures, since the spores tended to remain on the surface of the medium and form a pellicle more readily; the starch was gradually decomposed by the growing organism. No reducing sugar was ever demonstrated in the starch media; either the starch was hydrolyzed to the dextrin stage

and directly assimilated by the organism, or it was broken down to compounds not readily demonstrated, or the sugar was consumed as soon as formed. Only limited acid production by the organism was demonstrated in the above media. A comparison of the three best carbon sources, with tryptone (0.5 per cent) as the source of nitrogen, upon the production of streptothricin in shaken and in stationary cultures, is brought out in figure 2. The shaken cultures gave much greater activity in a shorter period of time.

A detailed study of the growth of the organism, as measured by dry weight of mycelium and production of streptothricin, using shaken cultures, pointed to a correlation between the two, as illustrated in figure 3. This parallelism tends to indicate that the formation of the antibiotic substance is a function of the growing organism, under favorable conditions, and not of the autolyzing cells.

TABLE 3

Influence of depth of medium upon the production of streptothricin
Starch-glycine medium, stationary cultures

VOLUME OF MEDIUM PER LITER FLASK	INCUBATION	ACTIVITY	
		<i>E. coli</i>	<i>B. subtilis</i>
ml.	days	units	units
100	6	100	>300
200	6	30	250
300	6	10	30
100	9	100	>300
150	9	75	300
200	9	30	250
250	9	20	200
300	9	20	150
300	17	20	100

Influence of aeration upon the production of streptothricin

Among the various factors influencing the formation of streptothricin, none is more important, in addition to the essential nutrients, than the air supply. This is illustrated by the following experiment. Different volumes of the starch-glycine agar-free medium were placed in 1 liter Erlenmeyer flasks, so as to obtain varying depths of medium. The flasks were inoculated with a spore suspension of an active strain of the organism and incubated under identical conditions, in a stationary state. The antibiotic activity of the cultures was measured after varying periods of incubation (table 3). An inverse proportion was obtained between the volume of the medium in the flask and the production of streptothricin. The shallowest layer, 100 ml. per liter flask, gave as high activity as that obtained in shaken cultures using the same medium.

Various other surfaces, such as cotton, paper, peat and straw, were compared for the growth of *A. lavendulae* and for the production of the streptothricin. These substrates were saturated with the glucose-tryptone medium. The best activity was obtained on the cotton medium. However, none proved superior

to, if as good as, the ordinary starch-tryptone or starch-glycine medium, either in very shallow stationary layers or in shaken cultures.

Influence of temperature and other factors

The temperature of incubation of the cultures is known to have an important effect upon the production of antibiotic substances by microorganisms. This was also found to hold true of streptothricin, as brought out in table 4. The highest activity was obtained at the lowest temperature of incubation, namely at room temperature. A more detailed study was made of the production of streptothricin at 23 and 28°C., using varying incubation periods and sources of nitrogen; the lower temperature invariably gave the greater antibiotic activity.

TABLE 4
Influence of temperature upon the production of streptothricin
Stationary cultures

TEMPERATURE OF INCUBATION	PERIOD OF INCUBATION	ACTIVITY	
		<i>E. coli</i>	<i>B. subtilis</i>
°C.	days	units	units
20	7	15	200
20	10	75	500
20	14	100	500
28	7	50	200
28	10	50	300
28	14	60	250
30	7	30	150
30	10	50	200
30	14	25	75
37	7	10	20
37	10	<10	5
37	14	<10	<10

A detailed study of the effect of reaction upon the growth of *A. lavendulae* and the production of streptothricin in tryptone-starch medium brought out the surprising fact that variations in pH of the original culture between 5.0 and 7.5 had very little effect. In the more acid cultures, there was a gradual increase in the pH value with the growth of the organism, due to the production of ammonia from the tryptone; in the media with the less acid reactions, there was at first a decrease in pH value, soon followed by an increase.

The addition of yeast extract to the standard tryptone-starch medium either had no effect at all or suppressed streptothricin production.

Correlation between growth of A. lavendulae and the production of streptothricin

Attention has been directed above to the close correlation between the growth of the organism and the production of streptothricin. The results of another experiment, reported in table 5, show that in stationary as well as in shaken

cultures, with tryptone or with glycine as sources of nitrogen, growth and activity reached a maximum and then declined, the maximum for the first preceding somewhat that of the second. Since the nitrogen in the dry mycelium varied between 7 and 9 per cent, growth may be expressed by the dry weight of the mycelium or by its nitrogen content. One must, therefore, conclude that the production of streptothricin is not a result of autolysis of the mycelium but is due to cell nutrition or to cell synthesis. This renders the mechanism of the production of this substance distinct from that of tyrothricin, for example, which is a result of autolysis of the bacterial cells, or of penicillin, which is produced at a much later stage of growth of the organism, namely, when it reaches an alkaline reaction.

TABLE 5
Growth of A. lavendulae and production of streptothricin
Tryptone-starch medium

AERATION	INCUBATION	STARCH LEFT	DRY WEIGHT OF MYCELIUM	NITROGEN IN MYCELIUM	ACTIVITY	
					<i>E. coli</i>	<i>B. subtilis</i>
	days		mg.	mg.	units	units
Shaken.....	2	+++			10	5
Shaken.....	3	+	225	18.2	10	50
Shaken.....	4	0	293	26.2	75	250
Shaken.....	6	0	231	17.3	100	300
Shaken.....	8	0			75	200
Shaken.....	12	0	142	9.6	30	50
Stationary.....	7	+++			50	200
Stationary.....	10	0*	235	18.8	50	300
Stationary.....	14	0*			60	250

* Small amount of starch left at bottom of medium.

The efficiency of utilization of the carbon and the nitrogen by *A. lavendulae* is very high. At the maximum growth stage, 65 per cent of the nitrogen in the glycine added to the medium became converted into actinomyces cell substance. Since as much as 330-350 mg. of mycelial growth was obtained from 1 gm. of raw starch, the efficiency of utilization of the carbon, considering the carbon content of the starch as well as of the glycine, is about 40 per cent.

Bacteriostatic spectrum of streptothricin

It has already been demonstrated (Waksman and Woodruff, 1942), by the use of crude culture filtrate as well as concentrated crude preparations, that streptothricin has a selective bacteriostatic effect against both gram-negative and gram-positive bacteria. Additional results are reported here, using a salt-free, purified streptothricin preparation (table 6); these results are definitely similar to those presented previously. The selective action is very striking. *B. subtilis* is by far the most sensitive organism to the action of streptothricin, *Bacillus mycoides*

and *Bacillus cereus* are less sensitive than most gram-negative bacteria. The same is true of the variation among the gram-negative bacteria, as illustrated by the two extremes, namely *Shigella gallinarum* and *Pseudomonas fluorescens*. Whereas *Sarcina lutea* is about as sensitive to streptothricin as *E. coli*, the ratio in sensitivity between the two against actinomycin is about 10,000:1.

TABLE 6
Antibacterial spectrum of purified streptothricin

ORGANISM	UNITS OF ACTIVITY	ORGANISM	UNITS OF ACTIVITY
<i>Escherichia coli</i>	100,000	<i>Bacillus subtilis</i>	750,000
<i>Aerobacter aerogens</i>	30,000	<i>Bacillus mycoides</i>	<10,000
<i>Pseudomonas fluorescens</i>	<10,000	<i>Bacillus cereus</i>	<10,000
<i>Shigella gallinarum</i>	300,000	<i>Staphylococcus aureus</i>	200,000
<i>Shigella dysenteriae</i> , 8712.....	100,000	<i>Sarcina lutea</i>	100,000
<i>Shigella dysenteriae</i> , 8708.....	100,000		
<i>Shigella dysenteriae</i> , 7424.....	30,000		
<i>Shigella paradysenteriae</i>	50,000		

DISCUSSION

Among the thirty or more antibiotic substances which have already been isolated from different groups of microorganisms, only four so far either have found practical application or show promise of such application, because of their low toxicity to animals and their *in vivo* activity against pathogenic bacteria. These are, in order of their discovery, 1. pyocyanase, a substance which has had a varied history and for which, at various times, many claims have been made but not always substantiated; 2. penicillin, which recently has found quite extensive practical application; 3. tyrothricin (gramicidin and tyrocidine), which has become recognized as an agent with certain definite potentialities; 4. streptothricin, a substance active not only against certain gram-positive but also against gram-negative bacteria.

These four substances have been isolated from cultures of different microorganisms, representing gram-negative non-spore-forming bacteria, fungi, gram-positive spore-forming bacteria, and actinomycetes, in the order listed above. These substances vary not only in origin, but also in chemical nature, solubility, and specific antibacterial activity, i.e., their respective bacteriostatic spectra. The limited information concerning their mode of action upon bacteria definitely suggests great variation in this respect as well. Because of these variations, one can easily understand why the substances should vary also in their activity in the animal body.

The latest addition to the list of active compounds, streptothricin, is one of a series of five substances which have so far been obtained from actinomycetes, a group of organisms containing a large percentage of forms with antagonistic properties. Streptothricin is a nitrogen-containing base. It is insoluble in ether and soluble in water. It has a low toxicity to animals. It is active both against

certain gram-positive and gram-negative bacteria. It has been isolated in a concentrated form, although it has not been crystallized as yet.

The presence of certain amino acids or a mixture of amino acids (polypeptides) in the medium is favorable to the formation of streptothricin. Although the organism grows well with NaNO_3 as a source of nitrogen, it produces little of the antibiotic substance. One may, therefore, conclude that streptothricin is largely formed from one or more of the amino acids. The fact that larger amounts of the active substance are produced in the presence of an additional carbohydrate, such as starch, maltose or glucose, does not detract from this conclusion. The carbohydrate may serve only as a nutrient for cell synthesis, whereas the nitrogen source contributes to the formation of streptothricin. The direct parallelism between the growth of the organism and the production of the substance serves to substantiate this theory.

Aerobic conditions are absolutely essential to the production of streptothricin. The results presented in this paper amply justify this conclusion. In spite of the fact that the organism may produce in stationary cultures a mass of growth on the bottom of the flask, comparatively little streptothricin is produced if no pellicle is formed.

Acid conditions of the medium do not interfere with the production of streptothricin; this may possibly be due to its basic character, since this substance forms salts readily and is isolated by removal in an acid solution.

Several actinomycetes were found to produce an antibiotic substance which was, on the basis of its selective antibacterial action, similar to streptothricin. It is not known as yet whether the differences observed in the production of this agent by the different organisms are rather quantitative in nature or whether there is actually a chemical difference in the nature of the substance produced by the various organisms.

SUMMARY

Different strains of *Actinomyces lavendulae* isolated from soil, dust or an active mother culture were found to vary in their ability to produce the antibiotic substance streptothricin.

The formation and accumulation of streptothricin is largely controlled by the conditions of nutrition of the organism and the aeration of the culture.

Streptothricin is formed largely when a protein digest or certain amino acids, namely, glutamic acid, glycine or asparagine, are used as sources of nitrogen. When glucose is used as the additional carbon source, a small amount of agar (0.25 per cent) was found to favor the growth of the organism and results in the maximum production of streptothricin. When starch is used in place of glucose, no agar is required. The starch is consumed rapidly, but no reducing sugar could be demonstrated.

Supplementary additions of yeast extract and similar materials had an injurious effect upon the production of streptothricin in stationary cultures.

Aeration was found to be one of the most essential factors in the production of streptothricin. Either very shallow layers must be used or the culture must be

kept in a well-aerated submerged state. In the case of stationary cultures, the formation of a surface pellicle is essential for the optimum production of the antibiotic substance.

An incubation temperature of 20–23°C. for 7–10 days proved to be optimum for the production of streptothricin in stationary cultures; for shaken cultures growing at 28°C., 2–4 days incubation was sufficient.

The reaction of the medium, ranging between pH 5.0 and 7.5, had little effect upon growth and streptothricin formation. With the less acid reactions, the culture medium first became acid, as a result of the growth of the organism, and then changed to alkaline.

A certain parallelism was found to exist between the growth of the organism and the formation of streptothricin. This antibacterial substance is a metabolic waste product, which is apparently produced from certain amino acids or polypeptides.

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NOTES

POLIOMYELITIS INDUCED BY INOCULATION OF TOOTH PULP CAVITIES

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Since there is still considerable uncertainty regarding the path by which the poliomyelitis virus reaches the central nervous system, it appears of interest to determine whether other routes beside the respiratory and alimentary tracts may serve as the entry portals. The recent report by Faber and Silverberg (1942) of the presence of the virus in the semilunar (Gasserian) ganglion suggested to one of us (M. S. A.) that the virus might enter the pulp chamber of a carious tooth with pulpal exposure and travel via the maxillary and mandibular divisions of the fifth nerve to the semilunar ganglion and thence to the central nervous system.

To test this hypothesis, the pulp chambers of the anterior teeth of three *Macacus rhesus* monkeys under nembutal anesthesia were exposed. The pulps were removed and a drop of 20 or 40 per cent suspension of the "Creach" strain of the virus was placed in each canal and the cavities sealed with "Plicene" (Central Scientific Co.) in the successful experiment and silver amalgam in the unsuccessful experiments. Two of the three animals inoculated with the use of silver the first or second time failed to develop paralysis during an observation period of 16 to 50 days. The negative results may have been due to the oligodynamic action of the silver fillings and the use of only a 20 per cent suspension of virus. The third animal was inoculated in a similar fashion with 20 per cent virus and the cavities sealed with Plicene. When paralysis did not develop after a three-week observation period, the animal was again inoculated with a 40 per cent suspension of virus and the cavities sealed with Plicene. Seven days later this animal showed tremors of the hind legs and all four legs were paralyzed on the following day. On the ninth day the monkey was sacrificed and histologic sections of the cord showed the characteristic pathology of poliomyelitis. The Gasserian ganglia showed marked cellular infiltration, some neurophagia and karyolysis of many of the ganglion cells.

It was considered desirable to publish this preliminary report since there is no evidence in the literature of poliomyelitis having been previously produced experimentally by inoculation of pulp canals. It is by no means implied that this experiment proves that poliomyelitis may be acquired under natural conditions in human beings by infection through carious teeth. However, we believe that these results do indicate a possible pathway of the virus which has not been hitherto considered.

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- FABER, H. K., AND SILVERBERG, R. J. 1942 Pathway of invasion in the *Cynomolgus* monkey after oral application of poliomyelitis virus. *Science*, **96**: 473-475.

THE USE OF "PYREX" BRAND FRITTED FILTERS IN BACTERIOLOGICAL WORK

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The application of sintered or fritted glass filters to bacteriological work was reported by Morton and Czarnetzky in 1937. The sintered glass filters available at that time were made of Jena laboratory glass. That glass is no longer available and it is very doubtful if filters of Jena glass will be available after the war. The constant interest in and the use of sintered glass filters for certain bacteriological work prompted an investigation to determine if filters manufactured from "PYREX" Brand Chemical glass #774 in this country are efficient in retaining bacteria.

Such filters were stated to have a maximum pore diameter of 1.3 to 1.9 microns and were designated UF (ultra-fine) by the manufacturer.¹ Filters of the Buchner type containing a 35 mm filtering area (40 mm disc) filtered 100 ml of distilled water in 7 to 9 minutes under a pressure of 660 to 690 mm of mercury. Fifty ml of a 1:10 dilution in broth of a 24 hour old broth culture of *Serratia marcescens* were filtered in 7 to 35 minutes by the same filters under similar conditions. All filtrates remained sterile during an observation period of one week.

To determine the length of time required for the test organism to penetrate the "PYREX" sintered disc, a filter was assembled so that sterile broth was in contact with both sides of the disc. The broth on one side of the disc was inoculated with *Serratia marcescens* and the broth on the other side of the disc subcultured periodically. The subcultures made after 7½ and 17 hours remained sterile, whereas those made at 24 hours sometimes showed the presence of the test organism.

The filters are cleaned as previously described and assembled in the conventional manner (Morton, 1938, Morton and Czarnetzky, 1937). The safest way to insure that all the acid cleaning fluid has been washed out of the filtering disc is to test the pH of the wash water.

Browne (1942) reported that unbuffered solutions became more alkaline when filtered through Seitz filters. That point has been verified with distilled water. The pH of distilled water is not changed when it is filtered through a clean "PYREX" sintered disc.

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¹ Obtainable from the Corning Glass Works, Corning, New York.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

EASTERN PENNSYLVANIA CHAPTER

ONE HUNDRED AND SIXTY-FIFTH MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY
BUILDING, MARCH 23, 1943, PHILADELPHIA, PA.

SOME RECENT DEVELOPMENTS IN THE STUDY
OF STAPHYLOCOCCI. *John E. Blair*. Hos-
pital for Joint Diseases, New York, N. Y.

While pigmentation and hemolysis of blood agar often accompany pathogenicity of staphylococci, they must be considered only as presumptive evidence of pathogenicity. The coagulase reaction is recognized as the property most closely associated with pathogenicity of the staphylococci. Its simplicity of performance makes it available to diagnostic laboratories as a means of distinguishing between pathogenic and non-pathogenic staphylococci.

It is suggested that the serologic classification of staphylococci may be valuable in epidemiologic studies of staphylococcal infections.

Toxigenic staphylococci produce characteristic clinical and cardiovascular responses when injected intravenously into

rabbits. These reactions are absent when nontoxigenic strains are similarly injected. Animals may be protected from these effects by the use of antitoxin.

In children and young adults toxigenic staphylococci produce an acute fulminating systemic disease which may be recognized by characteristic clinical symptoms. Chief among these are a rapid pulse rate, which is out of proportion even to the elevated temperature, and symptoms indicating irritation of the central nervous system and of the gastro-intestinal tract. The toxemia may be controlled by the use of adequate amounts of staphylococcal antitoxin, properly administered.

In persons over the age of about 30 years there exists a certain degree of resistance to the systemic effects of toxin, and antitoxin therapy is not indicated.

ONE HUNDRED AND SIXTY-SIXTH MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY
BUILDING, APRIL 27, 1943, PHILADELPHIA, PA.

PENATIN, THE SECOND ANTIBACTERIAL SUBSTANCE PRODUCED BY *Penicillium notatum* WESTLING "77". *Walter Kocho-laty*, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pa.

Penatin, the second antibacterial substance produced by *Penicillium notatum*, Westling "77", is a protein, an enzyme which displays its antibacterial power only in the presence of glucose. The glucose is decomposed under formation of hydrogen peroxide and an acid, probably gluconic acid. Penatin is in great likelihood similar to, if not identical with, "Notatin" and "Penicillin B". Preparations of penatin have been obtained which will inhibit bacterial growth in dilutions up to and over 1:500,000,000. In addition to the various organisms affected by penatin and reported in previous papers, penatin is also bacteriostatic against *Klebsiella pneumoniae*, *Pasteurella pestis*, and *Vibrio comma* in dilutions

of 1:500,000,000. There are indications that penatin antagonizes bacteriophage and amoebae. *In vivo* experiments were conducted by Dr. E. L. Stubbs and Dr. I. Live on guinea pigs infected with about one million virulent *Brucella abortus* cells, and subsequent treatment with penatin. All injections were given subcutaneously. Large single doses, up to 500 mg, although tolerated by the animals proved to be ineffective, while repeated very small doses of penatin proved more effective. Four out of 16 guinea pigs treated in this way showed, after autopsy 6 to 9 weeks after the infection, no *Brucella abortus* cells detectable in inguinal lymph glands, spleen, liver, kidney, epididymis, and heart blood. The guinea pigs treated with penatin showed an average increase in weight per animal which was about twice the gain in weight of the non-treated controls. Due to the scarcity of the material only a modest experiment was conducted. A possible mechanism of the action of penatin *in vivo* was discussed

EFFECTIVE BLOOD AND SPINAL FLUID LEVELS OF SULFONAMIDE DRUGS IN THE TREATMENT OF MENINGITIS. *John A. Eiman and Harold W. Fowler*, Abington Memorial Hospital, Abington, Pa.

AN IMPROVED TECHNIC FOR THE CULTIVATION OF ANAEROBIC MICRO-ORGANISMS. *Harry E. Morton*, University of Pennsylvania, School of Medicine, Department of Bacteriology, Philadelphia, Pa.

The method is described in detail in a paper which will appear in the *Journal of Bacteriology*. Certain advantages of the method were discussed, such as safety and the presence of carbon-dioxide.

FACTORS INFLUENCING THE PROPAGATION OF INFLUENZA A VIRUS IN THE DEVELOPING CHICK EMBRYO. *Werner Henle and Gertrude Henle*, Children's Hospital, Philadelphia, Pa.

On prolonged incubation of eggs infected with the virus of influenza some inhibitor is formed in the allantoic fluid which on subculture may interfere with the propagation of the active virus. This inhibitor was

identified with inactive virus accumulating with increased incubation time.

In order to demonstrate the interference more clearly, attempts were made to inactivate the viable virus in the allantoic fluid. Heating to 56°C. for up to 1 hour or ultraviolet irradiation for 30 to 60 minutes usually inactivated enough of the virus to render such fluids innocuous for mice but sufficient concentrations of active virus were left to initiate some growth in the allantoic cavity of the chick embryo. When active virus in optimal concentration was inoculated into eggs either simultaneously with, or 3 hours following, inoculation of such partially inactivated fluids and the allantoic fluids collected after 48 hours of incubation, frequently no red-cell agglutinating agent was found and the active virus titer as tested in mice amounted to only a fraction of one per cent of the control fluids harvested from embryos following injections of normal allantoic fluid and virus. Similar interference experiments in mice indicated that the same phenomenon may be demonstrated in this species.

ONE HUNDRED AND SIXTY-SEVENTH MEETING, BIOLOGICAL LABORATORY, GUYOT HALL, PRINCETON UNIVERSITY, PRINCETON, N. J., MAY 15, 1943.

A joint meeting of the New Jersey, New York City and Eastern Pennsylvania branches. Abstracts of papers presented published under the proceedings of the New Jersey branch. *J. Bact.* 46, 109-111.

ONE HUNDRED AND SIXTY-EIGHTH MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, MAY 25, 1943, PHILADELPHIA, PA.

BACILLARY DYSENTERY. *Joseph Felsen*, 120 East 39th Street, New York, N. Y.

When the unclassified diarrheas are studied bacteriologically, the majority appear to be bacillary dysentery. Three to 10 per cent of patients with bacillary dysentery become carriers. They usually are "sick carriers". A clinical classification of the forms of bacillary dysentery based upon the bacterial type involved is not acceptable to the critical observer. Recognition of the appendicular, meningitic, pneumonic, arganulocytoid, constipated, asymptomatic and constipated forms is essential for adequate control. Boyd's classification of the *Shigella paradysenterias* appears to be the most comprehensive. Some strains still defy accurate classification. Change from S to R is generally associated with loss of

virulence. Besides the enteric and neurotropic toxins, there are probably arthritic, pneumonic and neurotropic fractions. Bacteriophage is of diagnostic and epidemiological value. The three-stage progression of pathology characteristic of acute bacillary dysentery is punctate follicular hyperplasia, punctate follicular necrosis, discrete and confluent ulceration on the first, second, and third days, respectively. Attention is directed to the use of indigenous strains for prophylactic vaccination and convalescent type specific human serum for curative therapy. Chronic ulcerative colitis and distal ileitis are forms of chronic bacillary dysentery. The ideal therapy for these chronic types is the prevention of the acute disease.

CHARACTERISTICS OF BUTYRIC ACID BACTERIA FROM OLIVES

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I. INTRODUCTION

The butyric acid bacteria have been associated with malodorous fermentations of olives for many years, although investigators have never recorded the isolation of such bacteria from abnormal olives, or studied the bacteria in any detail.

In the early stages of the butyric fermentation of olives the odor is distinctly that of butyric acid, or rancid butter. As the spoiling progresses the odors become more pronounced and less specific, and finally may result in a very malodorous stench. The butyric fermentation has been of common occurrence in green olives undergoing either the Spanish or Sicilian type pickling processes; to a lesser degree in olives held in salt brines prior to "ripe" pickling or in olives undergoing the "ripe" pickling process.¹

The butyric spoilage most commonly occurs during the primary stage of fermentation when an abundance of glucose and mannitol is present in the olive brines. At this stage the reaction (pH) of the brine is near the neutral point; the floral population is dominated by gram-negative and other bacteria commonly found in water and soil; the desirable lactic acid bacteria are in the minority; the butyric anaerobes gain the ascendancy, dominate the bacterial population and spoil the olives.

Hayne and Colby (1895) and Hayne (1900) were among the first to record spoiling of olives by the action of the "butyric ferment." Later Cruess and Guthrie (1923), and Cruess (1924; 1930), referred to butyric spoilage of olives. None of these authors studied the bacteria involved in the spoiling. Other references to this abnormality of olives were not found.

It is the purpose of this paper to characterize the anaerobic, butyric acid bacteria which have been found repeatedly in olives undergoing butyric fermentation.

II. EXPERIMENTAL

Data shown in table 1 were taken from analyses of Spanish type green-olive brines from which the butyric anaerobes were isolated. Most of the samples were collected from industrial fermentations while the butyric acid bacteria were still very active. Sample K came from a laboratory outbreak. Samples G1, G2 and M3 were collected from olives whose brines had been acidified with acetic acid in an attempt to arrest the spoilage.

¹ For a detailed discussion of olive pickling see Cruess, (1938), and Vaughn, Douglas and Gilliland (1948).

It will be noted that there was no uniformity in the chemical constituents of the brines. With the exception of the three acidified samples, the acidic constituents were low. Neither the non-volatile nor the volatile acid fractions were consistent with the degree of spoilage. The pH values of the non-acidified brines ranged from 4.6 to 7.2. The sodium chloride content was extremely variable. Normal brines, on the other hand contain from 0.7 to 1.25 grams of lactic acid per 100 ml.; they contain from 7.0 to 8.0 per cent salt and have pH values of 3.5 to 4.5.

TABLE 1
Analyses of Spanish type green-olive brines affected by butyric fermentation

SAMPLE NUMBER	NON-VOLATILE ACID AS GRAMS LACTIC ACID PER 100 ML. OF BRINE	VOLATILE ACID AS GRAMS BUTYRIC ACID PER 100 ML. OF BRINE	NaCl, GRAMS PER 100 ML. BRINE	pH
K	0.111	0.0528	2.62	5.0
D1	0.018	0.0088	10.55	5.25
D2	0.0313	0.0132	10.31	5.3
S1	0.135	0.251	0.917	4.6
S2	0.023	0.022	0.82	5.5
S3	0.045	0.106	8.75	4.9
L2*	0.081	0.097	0.328	5.3
L4*	0.090	0.361	0.491	4.8
E4	0.149	0.038	5.67	5.85
C2	0.315	0.273	7.25	5.9
H	0.018	0.009	3.92	7.2
G1	0.284	0.453	7.43	4.0
G2	0.363	0.418	7.60	3.9
M3	0.189	0.468	8.78	4.15

* Manzanillo variety, all other samples were Sevillano olives.

A. Isolation and purification of the bacteria

Portions of abnormal brine in quantities varying from 10 to 50 milliliters were added to sterile flasks containing sufficient sterile calcium carbonate to neutralize the acidity of the suspected brines. These preparations were heated to 80°C. (176°F.) and held for 30 minutes. All cultures were obtained by preliminary enrichment of samples of the heat-treated brines in corn-liver medium. The cultures thus obtained were purified by serial passage through tryptone crystal-violet glucose broth followed by serial plating on either liver infusion agar or tryptone glucose agar held under anaerobic conditions in evacuated vegetable (potato) tissue jars.

A total of 50 cultures of anaerobic, spore-forming, saccharolytic, butyric acid bacteria were isolated from 14 samples of abnormal olives. Control cultures used throughout the investigation included *Clostridium butyricum*, *Clostridium beijerinckii*, *Clostridium pasteurianum*, *Clostridium acetobutylicum* and *Clostridium tertium* received from Dr. Elizabeth F. McCoy and *Clostridium botulinum* (*Clostridium parabolulinum* Type A) obtained from Dr. Sanford S. Elberg.

B. Differentiation of the cultures

Primary differentiation of the bacteria was made on the basis of the type of sporulation. Specific allocation, following Spray (1939), was accomplished by testing the cultures for motility, liquefaction of coagulated egg albumen, blackening of brain medium, liquefaction of gelatin and fermentation (gas formation) of xylose, glucose, lactose, maltose, galactose, glycerol, adonitol, mannitol, salicin, inulin and starch. All significant fermentation tests were repeated at least two times at wide intervals. Particular attention was paid to the fermentation of starch, glycerol and mannitol in order to be sure of differentiation between the closely related species of the butyric group.

TABLE 2
Characteristics of butyric acid bacteria from olives

NUMBER OF CULTURES	DECOMPOSITION OF PROTEINACEOUS MATERIAL			FERMENTATION AS INDICATED BY GAS PRODUCTION											SPECIES ALLOCATION
	Coagu- lated egg albumin	Brain medium	Gelatin lique- faction	Xylose	Glucose	Lactose	Maltose	Galactose	Glycerol	Adonitol	Mannitol	Salicin	Inulin	Starch*	
Number of cultures showing positive reactions†															
19	0	0	0	10	19	18	18	19	0	3	19	18	3	0	<i>Clostridium beijerinckii</i>
13	0	0	0	13	13	13	13	13	13	0	11	13	9	7	<i>Clostridium multi- fermentans</i>
10	0	0	0	10	10	10	10	10	0	0	0	10	7	10	<i>Clostridium fallax</i>
7	0	0	0	7	7	7	7	7	0	0	7	7	4	7	<i>Clostridium butyricum</i>
1	1	0	1	1	1	1	1	1	0	0	1	1	0	1	<i>Clostridium aceto- butylicum</i>

* Insoluble corn starch free from substances capable of reducing Fehling's solution.

† All cultures incubated at 30°C.

All cultures were actively motile. The spore-forming cells were all distinctly swollen and the spores of all cultures were contained in a subterminal position inside the cell. The other differential reactions are shown in table 2. The most abundant species isolated was *Clostridium beijerinckii* (19 cultures). *Clostridium multi-fermentans* was represented by 13 cultures. *Clostridium fallax*, the third most abundant type, was represented by 10 cultures. Seven cultures were identified as *Clostridium butyricum*. Only one culture of *Clostridium aceto-butylicum* was found.

On the basis of the method of enrichment and purification, it is obvious that the non-proteolytic anaerobes would predominate. However, it is interesting to note that *Clostridium beijerinckii* was most frequently encountered among the anaerobic saccharolytic butyric-type bacteria, whereas no cultures of *Clostridium pasteurianum* were isolated. It is also interesting that *Clostridium fallax*

was encountered so frequently (10 strains, classified on the basis of sugar fermentations and difficulty with which the sporulation was demonstrated) inasmuch as this organism has been associated with war wounds and appendicitis in the past.

C. Maximum salt tolerance

The spores of the butyric acid bacteria are widely distributed in nature and it is probable that most barrels, tanks and vats of olives contain at least a few spores of these undesirable bacteria in their brines. Preservation of green olives by fermentation is dependent in part upon the concentration of salt and the acidity in the brines.

To test the resistance of the butyric anaerobes to salt the cultures were grown in liver infusion medium with 0.25 per cent agar at 30°C. When the cultures were growing vigorously they were inoculated into the liver infusion agar containing sodium chloride in concentrations of 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0

TABLE 3
Resistance of butyric acid bacteria to sodium chloride

ACTERIA	NaCl, GRAMS PER 100 ML.*					
	1	2	3	4	5	6
	Number producing gas					
<i>Clostridium beijerinckii</i> (19 strains)	19	17	15	11	7	0
<i>Clostridium multif fermentans</i> (13 strains)	13	13	12	8	4	0
<i>Clostridium fallax</i> (10 strains)	10	10	7	4	0	0
<i>Clostridium butyricum</i> (7 strains)	7	7	7	5	4	0
<i>Clostridium acetobutylicum</i> (1 strain)	1	1	1	1	1	1
Total	50	48	42	29	16	1

* Contained in liver infusion.

per cent (grams NaCl per 100 milliliters of liver infusion). The cultures were first inoculated into the liver agar containing 1.0 per cent NaCl. When gas production was observed in this test medium a portion of the culture was inoculated into liver agar containing 2.0 per cent sodium chloride. This adaptation technique was used until the cultures failed to show visible signs of growth or gas production. This means of adaptation gave consistent results whereas, if the cultures were inoculated directly into liver agar or olives in brine containing various concentrations of salt, growth was erratic.

Results of this experiment are shown in table 3. All of the cultures grew in the presence of 1.0 per cent salt, and most of them tolerated 2 and 3 per cent salt. Twenty-nine of the 50 test cultures grew and produced gas in the presence of 4.0 per cent salt; 16 tolerated 5.0 per cent salt. Only 1 culture (*Clostridium acetobutylicum*) produced gas in the presence of 6.0 per cent salt. These results correspond with the observations recorded for *Clostridium butyricum* by Matsushita (1902) and Hof (1935).

It is possible that in natural surroundings the butyric acid bacteria may grow

in the presence of somewhat higher concentrations of salt. However, in the olive industry experience has shown that a concentration of 7.0 to 8.0 per cent salt in the brines of fermenting olives is sufficient to prevent this spoilage. Furthermore, the spoilage is most common with the Sevillano variety of fruit, where, because of the nature of the olives, the concentration of salt in the brines must be increased slowly to a final concentration of 7.0 to 8.0 per cent sodium chloride to prevent salt shrivel of the olives.

D. Minimum pH tolerance

It is known that some of the butyric acid bacteria are capable of growing in rather acid surroundings (Townsend, 1939; Spiegelberg, 1940, and others). The cultures isolated from olives were tested for pH tolerance by using 24-hour liver-infusion broth cultures which were inoculated into tryptone glucose broth medium with sodium thioglycollate adjusted to pH 7.0. When the inoculated

TABLE 4
Tolerance of butyric acid bacteria to various pH values in glucose media

BACTERIA	pH OF MEDIUM						
	7.0	6.0	5.4	5.0	4.8	4.5	4.3
	Number producing gas from glucose						
<i>Clostridium beijerinckii</i> (19 strains)	19	17	17	9	5	1	0
<i>Clostridium multi fermentans</i> (13 strains).	13	13	13	13	13	7	0
<i>Clostridium fallax</i> (10 strains)	10	10	10	10	9	8	0
<i>Clostridium butyricum</i> (7 strains)	7	7	6	5	5	1	0
<i>Clostridium acetobutylicum</i> (1 strain)	1	1	1	0	0	0	0
Total	50	48	47	37	32	17	0

test medium showed vigorous gas formation this culture was used for inoculation of the tryptone medium adjusted to pH 6.0. This adaptation technique was used until the minimum pH value for growth and gas production from glucose was reached. All test inoculations were incubated for 3 weeks at 30°C. before recording negative results for gas production in the medium with the lowest pH value.

The media were adjusted to the various pH values with buffers by adding 5 milliliters of 0.2 M Na_2HPO_4 , and 0.1 M citric acid in appropriate mixtures to 5 milliliters of double-strength tryptone glucose broth to give the desired pH after sterilization. The media contained 1.0 per cent tryptone, 0.5 per cent glucose and 0.05 per cent sodium thioglycollate plus the buffers. Final pH values after sterilization were 7.0, 6.0, 5.5, 5.0, 4.8, 4.5 and 4.3 as determined by the glass electrode.

Results of this experiment are shown in table 4. All of the cultures grew well in the medium adjusted to pH 7.0. Most of the cultures grew and produced gas in the media at pH 6.0 and pH 5.5 (48 and 47 cultures respectively). At the

lower pH values the effect became more striking. Only 37 cultures produced gas at pH 5.0 and 32 at pH 4.8. When the reaction was reduced to pH 4.5 only 17 cultures produced gas from the glucose and at pH 4.3 activity of all cultures had ceased. On the basis of these results the cultures of *Clostridium multifementans* and *Clostridium fallax* were most tolerant and those of *Clostridium beijerinckii* were least tolerant to the effect of low pH.

Cultures of *Clostridium pasteurianum* isolated by Townsend (1939) were capable of growth in pear juice with pH values of 3.6 to 4.0. Spiegelberg (1940) reported other cultures of *Clostridium pasteurianum* which caused spoilage of canned pineapple with a pH value of 4.2. As seen from table 1 some of the strains of butyric anaerobes isolated from olives came from brines with pH

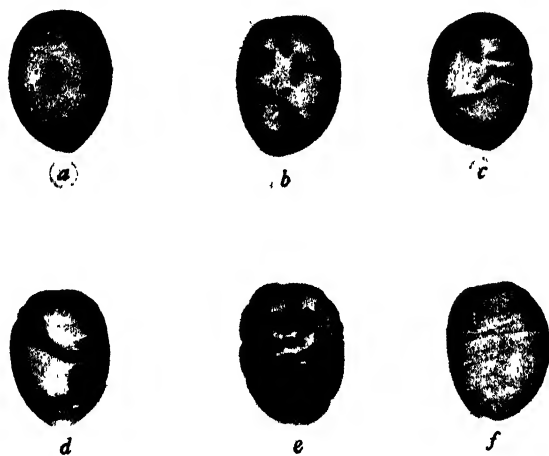


FIG. 1. GAS-POCKET FORMATION IN INOCULATED OLIVES

(a) Uninoculated control; (b) inoculated with *Aerobacter aerogenes*; (c) inoculated with *Escherichia coli*; (d) inoculated with *Aerobacillus polymyxa*; (e) inoculated with *Clostridium butyricum*; (f) inoculated with *Saccharomyces cerevisiae* var. *ellipsoideus*. (All olives; Mission variety; sterilized by intermittent steaming and incubated before inoculation.)

values as low as 3.9 and 4.0. In the case of the olives it is probable that the final pH resulted from added acid for it is known that those samples were acidified in an attempt to arrest the spoilage. Experience in the olive industry has shown that, if fermentation of the fruit is directed until the pH of the brine reaches pH 4.0, butyric spoilage is eliminated.

E. Gassy spoilage caused by the butyric acid anaerobes

Because of the malodorous fermentation of olives caused by the butyric anaerobes, their ability to produce gas-pockets in the flesh of infected olives has been overlooked. However, as shown in figure 1 olives inoculated with a culture of *Clostridium butyricum* became completely honeycombed with gas fissures. All of the cultures of butyric acid bacteria produced many gas-pockets in contrast with the coliform bacteria and *Aerobacillus polymyxa* which caused only a few large fissures to form in the fruit. It is believed that all bacteria which decom-

pose sugar with the liberation of sufficient hydrogen gas may cause gas-pocket formation in olives if conditions are favorable for their growth in the olive brines. Carbon dioxide is appreciably more soluble than hydrogen. It is assumed therefore, that the carbon dioxide is dissolved in the brine whereas the hydrogen, on the other hand, accumulates, eventually to form enough pressure to rupture the olive tissue and form gas-pockets.

Lactic acid bacteria (species of *Leuconostoc* and *Lactobacillus*) abound in olive brines, as do yeasts, yet they do not form noticeable gas-pockets in olive tissue. These microorganisms produce significant quantities of carbon dioxide, but do not liberate hydrogen.

III. DISCUSSION

Although no strongly proteolytic anaerobic bacteria were encountered in this investigation, it is to be stressed that under conditions existing in some olive brines dangerous types might develop, particularly when the pH value of the brine is above 4.5 and the salt content is less than 7.0 to 8.0 per cent sodium chloride. Furthermore, the methods of isolation used were more favorable for the saccharolytic butyric types. Nonetheless the attempted utilization of any olives affected by growth of anaerobic butyric acid bacteria is unwise for, although picklers commonly taste them, the danger of food poisoning must not be overlooked.

The absence of isolates of *Clostridium pasteurianum* undoubtedly is fortuitous. Townsend (1939) has isolated this organism from California produce. However, the particular abundance of the *Clostridium beijerinckii* type as compared with the number of true *Clostridium butyricum* isolates is probably an indication of the close relationship between *Clostridium butyricum* and the other saccharolytic, non-proteolytic² butyric acid bacteria at present recognized as distinct species. If, as indicated by Spray (1939), the closely related species are in reality varieties of *Clostridium butyricum*, then the whole group of anaerobic, non-proteolytic butyric species will be recognized as even more closely related than at present. Under such conditions the number of isolates of the *Clostridium fallax* type found in this study would not be unexpected even though all previously isolated cultures were of pathological origin.

IV. SUMMARY

The characteristics of 50 saccharolytic, butyric anaerobes isolated from samples of malodorous olives are given. These bacteria all caused deterioration of olives when suitable conditions for growth were maintained in olive brines. The species *Clostridium beijerinckii*, represented by 19 strains predominated among the cultures isolated. *Clostridium multi fermentans* was represented by 13 strains. Ten cultures were identified as *Clostridium fallax*; 7 as *Clostridium butyricum* and only 1 culture as *Clostridium acetobutylicum*.

The differential characters for the species isolated from olives were in good

² Non-proteolytic in the sense that gelatin is not liquefied; and that brain, heart or other meat or egg white and milk is not digested.

agreement with the descriptions previously recorded. Resistance to sodium chloride was variable. Tolerance to increasing acidity in the medium also was variable. The one culture of *Clostridium acetobutylicum* was most resistant to sodium chloride. The isolates of *Clostridium multifementans* and *Clostridium fallax* were most tolerant to low pH. All of the cultures grew much better in glucose media having pH values near neutrality (pH 7.0), and with less than 1.0 per cent sodium chloride in the media.

V. ACKNOWLEDGMENTS

The authors express their appreciation to Dr. Elizabeth McCoy for her helpful suggestions on differentiation of the bacteria, and to Professor W. V. Cruess for his kindly interest and assistance in collection of representative samples of olives.

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STUDIES ON THE NUTRITION OF DIM AND BRIGHT VARIANTS OF A SPECIES OF LUMINOUS BACTERIA

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A variant form of *Achromobacter fisheri* was frequently observed which differed from the original stock in a number of ways: namely, the colonies developed a conspicuous yellow pigment, the luminescence was more brilliant and lasting, and growth was more profuse than in the original stock. Self photographs of the two strains are shown in figure 1. It is evident that the variant was able to make more effective use of the nutrients provided in the medium than was the original stock. The experiments embodied in this paper were performed to determine the reason for the difference between the two strains.

Little is known concerning the chemical activities of luminous bacteria beyond the fact that they all grow well on complex media such as peptone or peptone-glycerol; that acid is produced in abundance from the various carbon sources, even under aerobic conditions (Hill, 1928); and that some will grow in inorganic media with a single simple organic source of carbon (Doudoroff, 1942).

The tendency for cultures of this species to become acid, coupled with the known sensitivity of their luminescence to acid, suggested that the difference between the two strains here studied might be (1) merely a difference in acid tolerance or (2) a difference in the rate of utilization of the acids formed as intermediaries in the decomposition of the complex nutrients. If the variant is better able to withstand acid than the original stock, utilization of the available nutrients might continue in the former case at a pH which would stop such usage in the latter. Thus, growth and luminescence might continue longer. If the variant is better able to utilize the acid intermediaries, it would also, by virtue of this fact, continue its activity for a longer time. The following analysis shows that both factors apparently play a part.

MATERIALS AND METHODS

Methods for the culture and preparation of suspensions of the bacteria and for the determination of their respiration and luminescence have been described elsewhere (Giese, 1941). The density of suspensions was measured with a densimeter of the type developed by Longworth (1936). The pH was in all cases determined with a Beckman glass electrode. All cultures were grown and experiments conducted at approximately 25°C.

The agar plates used for growth of the bacteria contained 1 per cent glycerol, 0.6 per cent peptone, 0.2 per cent yeast extract, 0.2 per cent beef extract, 1 per cent CaCO_3 , 2 per cent agar and 3 per cent NaCl. Liquid cultures were grown in 0.1 per cent peptone, 1 per cent glycerol and 3 per cent NaCl when no buffering was desired. The same nutrients with 1.5 per cent NaCl and M/8 solution of

the appropriate buffer were used when studies were to be conducted with liquid cultures at a given pH. Phosphate buffers were used in all cases.

In tests on the utilization of the organic acids for respiration, M/4 solutions of the acids neutralized with NaOH or NaHCO₃ were used in the side arms of the Warburg vessels giving, on dilution, approximately M/40 acid in the culture. Fresh solutions of all organic materials were made up for each series of experiments to minimize contamination.

Since for testing the utilization of various nutrients it is desirable to have bacteria containing relatively little stored material, cultures one day old were in each case washed from the plate in sea water and were centrifuged and suspended in fresh sea water and kept overnight in a refrigerator at about 5°C. This procedure was found by preliminary tests to exhaust the stored nutrients as effectively as aeration. On the following morning the cultures were again centrifuged and washed and suspended in buffered NaCl. For luminescence studies and in a few other cases mentioned in the text, 18–24 hour cultures were used.

EXPERIMENTAL

To determine the relation between pH and growth, bacteria of both strains were inoculated into liquid cultures buffered over the pH range 5.0 to 8.0 with peptone and glycerol as nutrients. It was found that the variant (hereafter designated Y) grew from 5.5 to 8.0 while the original stock (hereafter designated W) grew from 6.5 to 8.0. Growth in all cases was slight in the more acid cultures but no quantitative determinations were made. If Y grows better under more acid conditions than W because of its ability to utilize nutrients under acid conditions, its respiration should be greater than that of W under these conditions.

The relation between pH and respiration, occurring in the absence of nutrients in the medium and therefore presumably on the nutrients stored in the cells, was studied first. The respiration was found to increase with rise in pH as shown in figure 2. There is no essential difference between the respiration of the two strains over the pH range tested.

The respiration in the presence of nutrients in the suspension medium was therefore tried. It was found that neither Y nor W was capable of making effective use of peptone as the only organic nutrient at pH 6.0, for the respiration was little greater in presence of peptone than in its absence. An increase in usage occurred as the pH rose. At pH 7.5 the utilization of peptone was considerable and approximately equivalent for both strains. The data are summarized in figure 3. The difference in acid tolerance must reside in some property other than a difference in peptone utilization under acid conditions. However when the two strains are compared on the basis of the proportional increase in respiration over the endogenous rate on addition of peptone, Y has the advantage, the increase being roughly four to five-fold, whereas for W it is only a little over threefold for most of the pH range. Unfortunately different experiments are not completely consistent, indicating that the state of the bacteria may influence this response.



FIG. 1. PHOTOGRAPHS OF COLONIES OF BACTERIA TAKEN WITH THEIR OWN LIGHT

The bright colonies are the yellow variants, shown to be glowing brightly and still growing. Note that colonies of W are luminous, at least around the edges when they lie near Y; the side closest to Y in such cases is brightest

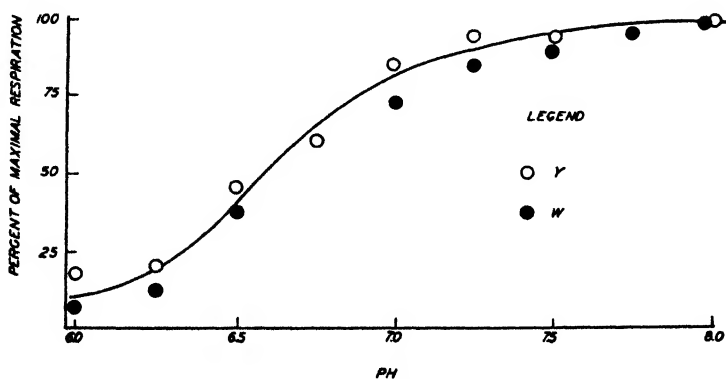


FIG. 2. THE RELATION BETWEEN pH AND ENDOGENOUS METABOLISM

Each of the points is the average of three separate experiments

In the following series of experiments it was found that there was a considerable difference in the utilization of glycerol by the two strains. Both Y and

W were able to use glycerol better, the higher the pH; for Y, considerable usage occurs over the range 6.5 to 8.0, for W, over the range 7.25 to 8.0. The experiments are summarized in figure 4. These experiments demonstrate that Y is better able to obtain energy for its respiration at a low pH than is W, which accounts in part for the ability of Y to grow under relatively acid conditions.

When either Y or W is supplied with glycerol as the only substrate, the unbuffered medium rapidly becomes acid, in both cases falling to about pH 6.0 in one-half hour, 5.5 in one hour and 5.0 in 7 hours. If the only difference between the two strains is the ability of Y to continue to use glycerol longer than W because of its lesser sensitivity to acid, one might expect that Y would merely continue to grow longer than W but that no other change in the medium would occur. Tests however disclosed that whereas agar plate cultures of W became and remained acid, those on which Y was growing became alkaline. Thus, even

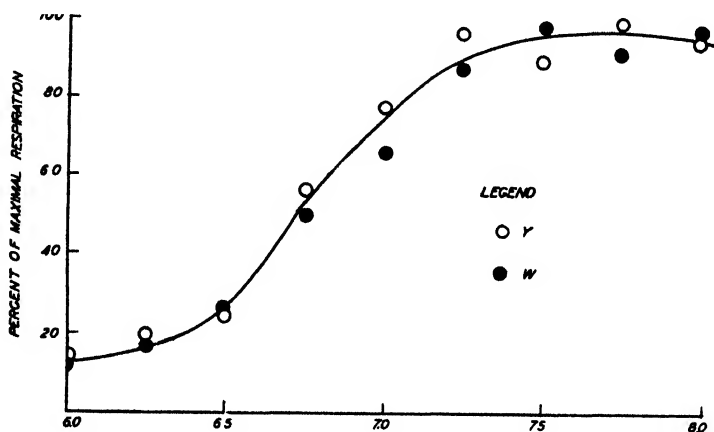


FIG. 3. THE RELATION BETWEEN pH AND PEPTONE UTILIZATION AS DETERMINED BY THE RATE OF RESPIRATION AT DIFFERENT pH VALUES
Averages of 4 experiments

on a plate on which W was streaked on one side, Y on the other, the pH was found to be about 6.0 on the extreme W side, about 7.5 to 8.0 on the extreme Y side. Also, in liquid cultures containing peptone and glycerol the pH of W cultures fell to 5.0 in four days, at which time growth and luminescence ceased, whereas cultures of Y became only slightly acid just after inoculation and later became somewhat alkaline. The results are given in figure 5. In these cultures the titratable acidity in cultures of W was twice that of Y on the third day after inoculation. It is therefore apparent that mere ability to use glycerol at a low pH is not the only difference between Y and W and that Y is either using the acids produced from glycerol or is neutralizing these acids with metabolites resulting from peptone metabolism.

To determine if the latter is true, the two strains were grown in liquid culture on peptone as the sole organic source. No difference in pH or in titratable

acidity was found between the two (fig. 5). Both Y and W produce alkali which, according to Hill (1928), is probably ammonia. The similarity in utilization of peptone in respiration by these two strains has already been pointed out (fig. 3).

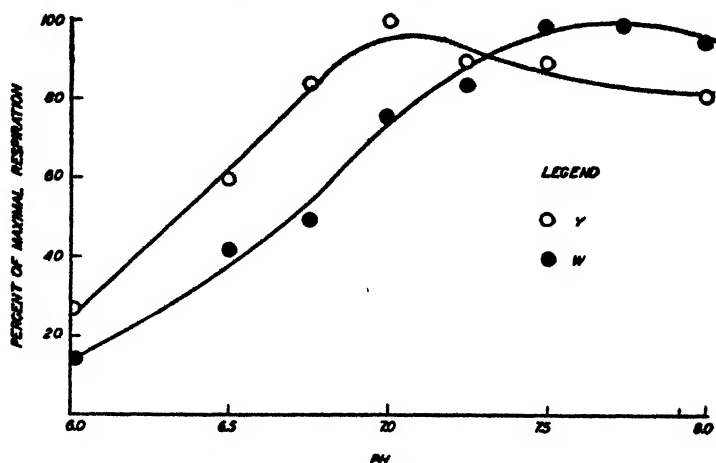


FIG. 4. THE RELATION BETWEEN pH AND RATE OF UTILIZATION OF GLYCEROL
Averages of three experiments

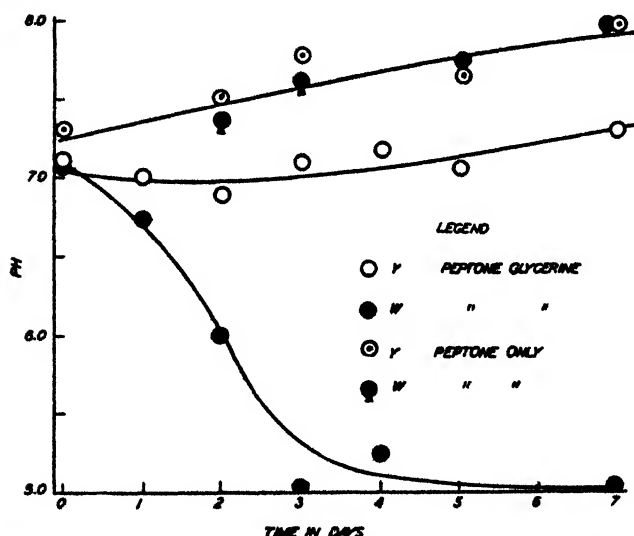


FIG. 5. THE CHANGE IN pH OF UNBUFFERED CULTURES CONTAINING IN ONE CASE PEPTONE ALONE, IN THE OTHER PEPTONE AND GLYCEROL
Average values

In the presence of glycerol it is possible that a difference in utilization of peptone by the two strains might occur.

The various experiments discussed above suggest that Y is better able than W to use acids produced from glycerol. It would therefore be interesting to determine which acids are used and to what extent. Unfortunately the inter-

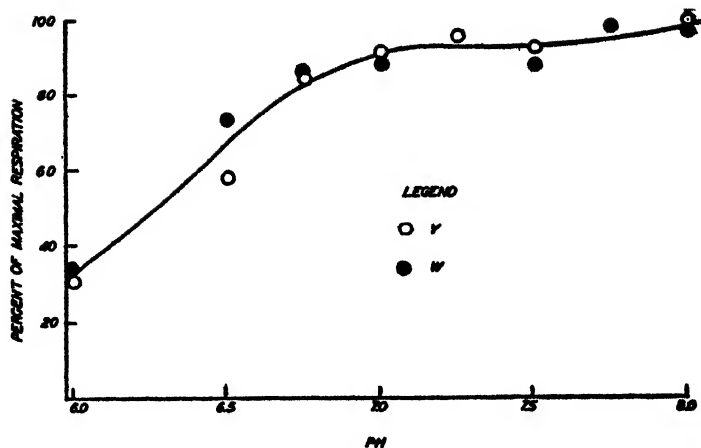


FIG. 6. THE RELATION BETWEEN RATE OF UTILIZATION OF PYRUVIC ACID AND pH
Averages of three experiments

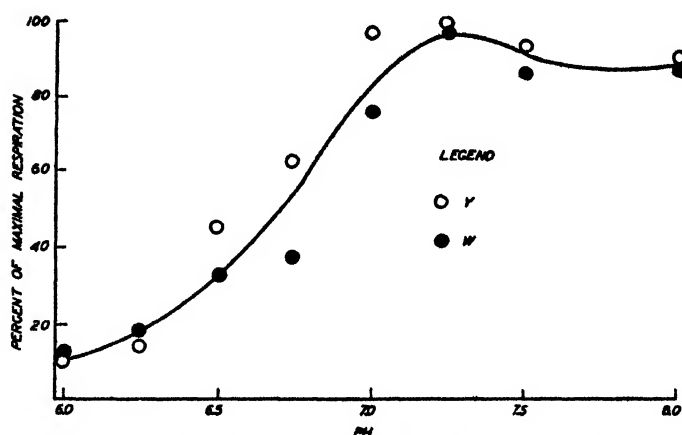


FIG. 7. THE RELATION BETWEEN THE RATE OF UTILIZATION OF SUCCINIC ACID AND pH
Averages of three experiments

TABLE 1

Relative rate of utilization of organic acids and peptone by Y and W strains of Achromobacter at pH 7.0

ACID	Y	W
Malic.....	2.4	1.9
Maleic.....	1.3	1.1
Fumaric.....	3.5	1.9
Succinic.....	3.9	1.9
Pyruvic.....	4.5	3.0

The figures were obtained by dividing the respiration in M/40 organic acid by the endogenous rate for an equivalent time. Glucose gives a value 15 times the endogenous rate. The usage varies with the state of the bacteria. Bacteria kept too long without nutrient do not respond.

mediary metabolism of these bacteria has not been adequately studied; however, on the basis of a survey of utilization of organic acids by microorganisms (Stephenson, 1939, pp. 190-197) a number of acids were chosen and tested. It was found that acetic, oxalic, lactic, tartaric, and citric were little if at all used, whereas maleic, malic, fumaric, succinic, and pyruvic were metabolized. In the utilization of the latter acids there were observed two differences between Y and W: (1) Y is able to use salts of some acids in a more acid medium than W; (2) Y is able to use some of them to a greater degree than W at a given pH. The data are summarized in table 1 and figures 6 and 7. In his recent study Doudoroff (1942 b) has shown that certain luminous bacteria produce fumaric, acetic, lactic and succinic acids, among other products in their anaerobic sugar dissimilation. If the species used here produces the same intermediate products it is surprising that Y in the presence of air is unable to use lactic and acetic acids.

DISCUSSION

The experimental data presented enable one to explain some of the differences between the dim and bright strains of *Achromobacter fisheri*. Both produce acid from glycerol, but since Y is better able to utilize glycerol even under acid conditions, it continues to grow after W has stopped. Secondly, since Y is able to use certain organic acids more effectively than W under acid conditions, it grows even after the supply of glycerol may have been depleted. Colonies of Y grow larger than colonies of W when the two are grown on the same plate as shown in figure 1. The more intense and continued luminescence of Y may also be in part due to the more efficient utilization of available nutrients by this strain.

The appearance of the bright variant Y is another example of the general tendency of the bacteria to adapt themselves to varied conditions (for references see Stephenson, 1939, ch. 11). The Y variant appeared frequently in liquid cultures of the original strain. In such cultures, in the absence of a buffer, the medium becomes acid rapidly and the appearance of a strain better able to tolerate the acid conditions is an adaptation. When the original strain was grown on agar plates and transfers were made every few days such changes were not observed. It should be pointed out that the degree of adaptability of the bacteria is limited. In unbuffered liquid cultures supplied with peptone and glucose the pH fell to about 4.9 or even lower in three to four days. Almost invariably the bacteria were killed, for growth seldom occurred when buffered nutrient medium was inoculated from such cultures. The bacteria were unable to adapt, either to the low pH or some other unfavorable conditions in the medium.

The mechanism by which the variation in *Achromobacter* occurs has not been investigated but since the Y strain appeared only several days after inoculation it seems probable that the change was a selection of naturally occurring variants such as those described in *Escherichia coli* by Massini (1907) rather than by a change in enzymes already present as described by Dubos (1940). Y was found to occur frequently in liquid cultures. The reversal to W was also found frequently on old plates and in old liquid cultures. Thus, on a plate 22 days old

containing only a small number of colonies, one large colony of Y was found to have five partial sectors of W.

A variation of the type Y to W in the production of dim mutants has been noted several times before, consequently it has been considered occasionally necessary in experimental work with luminescence to reisolate a brilliant strain from old cultures to be used as stock. Doudoroff (1938) investigated the nature of the dim mutants obtained in his cultures. He found that if these were grown in the presence of riboflavin and suspended in riboflavin solutions they equaled the brilliant strains in luminescence. This seemed an attractive explanation for the phenomenon described in this paper since the brilliant strain produces yellow pigment abundantly and the pigment diffuses into the medium, is soluble in water and fluoresces in the ultraviolet, suggesting riboflavin.¹ On this basis the W strain lacking the pigment might be considered dim because it is unable to produce the required riboflavin. However, when W was grown in the presence of extra riboflavin and suspended in solutions enriched with it, there was no essential change in luminescence. Secondly, the difference in pigmentation between the strains was found to be incidental for if both strains were grown on plates strongly buffered with phosphate salts at pH 8.0, both developed a pigment similar in color, neither developed pigment in acid cultures at first, Y developed such pigment when the cultures became alkaline.

The difference in luminescence of W and Y is apparently not an incidental one, due merely to pH and better availability of nutrients, for even when both strains are suspended at the same pH and adequate nutrient is supplied the luminescence of Y is 4 to 5 times greater than that of W. There is therefore some difference in the enzymatic system which controls luminescence. While it is possible that this difference is independent of the variation in sensitivity of the respiration to pH in the two strains, it is also possible that a fundamental alteration in some part of the enzymatic system underlies both.

It is interesting to point out one final difference between the two strains which might indicate some such fundamental change. It was found that when cultures of Y were suspended in solutions over a range of pH, precipitation of the bacteria occurred on the acid side of 6.8, whereas on the alkaline side very little or none occurred. Suspensions of W remained stable at all values of pH tried, except at or below 1.8 where denaturation may have occurred; suspensions also became clear at pH 10 due to cytolysis. It is clear that the surface properties of the two strains are different; Y acts as if it were charged negatively on the alkaline side of 6.8, W acts as if it were not so charged. For Y a covering with some protein is suggested, for W some other covering perhaps over the same underlying protein. Whatever the exact nature of this difference it indicates a characteristic modification of the cell properties with the variation from W to Y.

SUMMARY

1. A brilliant variant was observed to appear in liquid culture of *Achromobacter fischeri* when the medium was allowed to become acid. The luminescence of this form was 4 to 5 times that of the original strain under favorable conditions.

¹ W. J. van Wagtenonk, unpublished data.

2. On ordinary agar plates with calcium carbonate buffer, the variant developed a bright yellow-brown pigment, whereas the original strain did not. On buffered plates both strains developed the pigment at pH 8.0, neither on acidified plates.

3. Peptone increases the respiration of both strains and the sensitivity of each to pH is similar in both cases.

4. The variant uses glycerol more effectively than the original strain under acid conditions.

5. Neither strain uses a number of organic acids, both use succinic, fumaric, malic and pyruvic acids readily. The increase in respiration obtained by adding the acid is greater for the variant strain than for the original.

6. The variant strain behaves as if the individuals were charged, for precipitation occurs on the acid side of pH 6.8; the original strain is not precipitated at any viable pH.

7. Dim mutants regularly occur in cultures of the bright strain under certain conditions.

8. The bright strain may be considered a variant which adapts the species to the acid conditions unfavorable to the original strain. The time required for the appearance of a population of variants suggests that not adaptive enzyme formation but selection of naturally occurring variants accounts for the change.

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CULTIVATION OF BRUCELLA FROM THE BLOOD¹

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A rather extensive experience in duplicating blood cultures from patients suspected of suffering from brucellosis has shown a lack of uniformity in results despite the use of identical methods. It was formerly our practice to inoculate 8 to 15 ml. of the patient's citrated blood into a flask containing about 60 ml. of Bacto-tryptose broth (Difco) which was incubated at 37°C. for 4 to 6 weeks. At weekly intervals about 10 ml. of the supernatant broth was pipetted off, centrifugalized, and the sediment streaked on a blood agar slant. Since the report of Castaneda, Tovar and Velez (1942), we have adopted their procedure of dividing the blood sample and inoculating equal amounts into tubes of broth. We have modified the subsequent treatment of these tube cultures, and the method reported here is considered to be simpler and to increase the percentage of positive cultures.

METHOD

One to 4 test tubes, each containing 10 ml. of Bacto-tryptose broth (Difco), are inoculated with 2 to 5 ml. samples of the patient's or animal's citrated blood specimen and incubated at 37°C. From time to time the tubes are inspected for the appearance of colonies at the blood-broth interface. Regardless of whether or not such colonies are apparent (fig. 1), 6 to 7 days after inoculation (before hemolysis clouds the interface) a small amount (about 0.1 ml.) of material from the surface of the blood-broth interface is removed with a pipette (fig. 2) and inoculated onto a Douglas agar blood slant. The supernatant broth is then removed by pipetting and usually discarded, although in some instances this has been centrifugalized and its sediment streaked on a blood agar slant. The remaining blood, which represents almost the entire initial sample, is then added to molten Bacto-tryptose agar (Difco) and plates are poured. These subcultures are incubated at 37°C. and inspected daily for growth which usually is apparent after 48 hours.

RESULTS

Twenty-four tests were made on 7 positive blood cultures (*Brucella suis*) from a patient suffering from acute brucellosis. Since it is not our purpose to discuss the persistence or recurrence of bacteremia during this individual's illness, only the results of the positive blood cultures are given. Of 18 tube cultures from which subcultures were made from the blood-broth interface, the supernatant

¹ This work was aided by Dr. Mary P. Dole Fellowship of Mount Holyoke College and the John and Mary R. Markle Foundation.

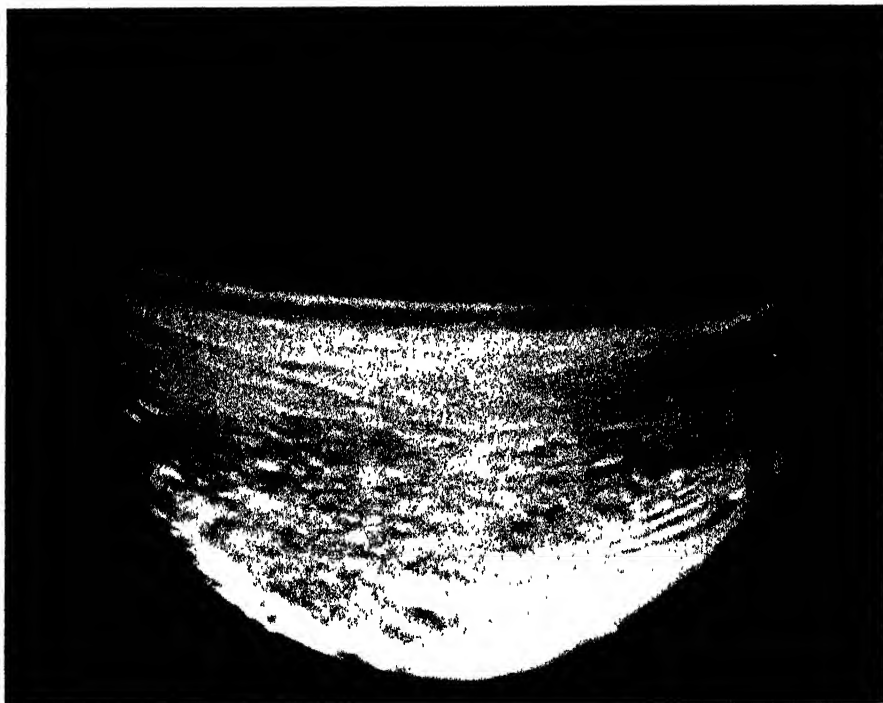


FIG. 1. PHOTOGRAPH OF A TUBE CULTURE TAKEN WITH CAMERA SLANTED DOWN UPON BLOOD-BROTH INTERFACE

The grayish area is the upper surface of the interface, upon which *Brucella* colonies are visible as raised white dots casting a faint shadow forward. The blood residue is not visible.

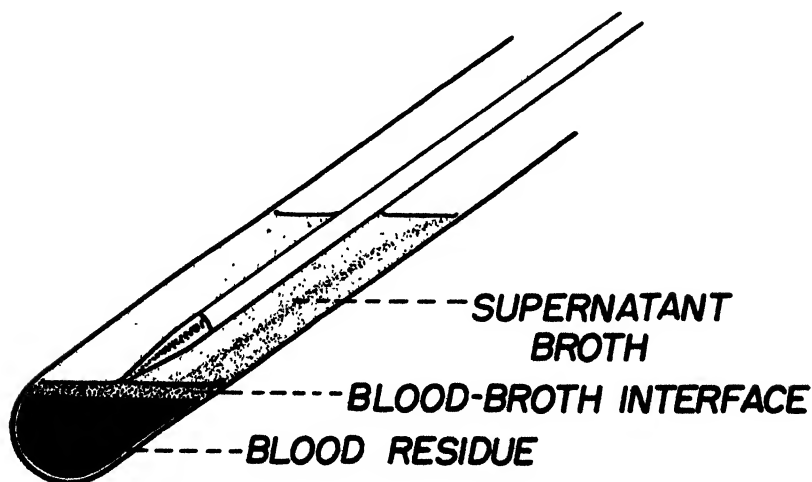


FIG. 2. DIAGRAM OF A TUBE CULTURE SHOWING A METHOD OF PIPETTING FROM BLOOD-BROTH INTERFACE

broth and the blood residue, the subcultures were all positive for *Brucella suis* in 7 instances and all negative in 5 instances. However, variable results were obtained from 6 culture tubes. In these subcultures *Brucella suis* was isolated in 2 instances from the blood-broth interface and supernatant broth but not from the blood residue. In the other 4 instances, the organism was isolated from the blood residue but not from the blood-broth interface or supernatant broth. Several tubes were used for each specimen of blood taken from this patient, but by no means was a positive culture obtained from every tube culture. Furthermore, had subcultures not been made from both the blood-broth interface and the blood residue, the percentage of positive cultures would have been materially lessened. Because the centrifugalized supernatant broth subcultures were never positive without either or both the blood-broth interface and blood residue subcultures being positive, in subsequent blood cultures from experimentally infected rabbits, the supernatant broth was discarded after its separation from the blood residue.

Thirty-seven positive blood cultures were obtained from 21 rabbits experimentally infected by the intracutaneous and intravenous routes with *Brucella suis* (strain ABF 36) for purposes of another experiment. The centrifugalized supernatant broths were usually discarded from these cultures, for reasons discussed in the preceding paragraph. Of the 91 tube cultures made on the 37 blood specimens, positive subcultures were obtained from both blood-broth interfaces and blood residues in 52 instances. In addition, 2 positive subcultures were obtained from the blood-broth interfaces when blood residue subcultures were negative, and 17 positive subcultures were obtained from blood residues when blood-broth interface subcultures were negative. The remaining 20 tubes yielded negative subcultures from both blood-broth interfaces and blood residues.

DISCUSSION

The lack of agreement shown here in the results of cultures of the same blood specimens indicates primarily that an ideal method for culturing *Brucella* has yet to be devised. Moreover, the very discrepancies illustrated here show the futility of attempts to duplicate such blood cultures, either for purposes of checking results or for comparison of methods, since identical cultural results may not be obtained from samples of a single blood specimen. In employing this method, subcultures must be made, regardless of whether or not growth is apparent in the tube cultures, for although colonies may not be visible at the blood-broth interface heavy growth may be obtained in subcultures. The necessity for subculturing both from the blood-broth interface and from the blood residue is shown by the lack of correlation between the results of these subcultures. It should be emphasized particularly that the plates poured from the blood residues must be subjected to the closest examination since overgrowth of such plates by *Brucella* results in very small, closely packed colonies which may easily be overlooked. This is true to a much greater degree than in overgrown poured plate cultures of other organisms which may appear negative on initial casual inspection. The

overgrown *Brucella* plates are often neither hemolyzed nor altered in any way except for a slight roughening of the surface, best observed by direct examination under a strong light. The organisms on such plates can be demonstrated readily by gram stain of the heavy but inconspicuous surface growth, and are identified by the usual criteria of lack of motility, failure to ferment sugars, and agglutination with specific antiserum.

Bacto-tryptose broth (Difco) has proven in our hands to be the most satisfactory medium now available for original isolation of *Brucella*, beef infusion broth (pH 7.1) being the next medium of choice. In our experience, liver infusion medium has not been adequate for isolation of brucella.

In sections of the country where *Brucella abortus* prevails cultures may be incubated under increased carbon dioxide tension.

SUMMARY

A simple method of blood culture for *Brucella* is described. The results of the employment of this method in an instance of human brucellosis and for studies of experimentally infected animals are given and discussed. The method is believed to give a higher percentage of positive cultures than obtained with previously described techniques.

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THE NOMENCLATURE AND CLASSIFICATION OF "THE ACTINOMYCETES"¹

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Since the publication by one of us (Waksman, 1940) of a system of classification of actinomycetes, considerable criticism was expressed in regard to the designation and position of the anaerobic pathogenic species, the cause of common actinomycosis in man and "lumpy jaw" in cattle. This type of organism was placed in the genus *Cohnistreptothrix* Pinoy, the generic name *Actinomyces* being reserved for the aerobic species forming aerial mycelium-bearing spores. This could be justified on the ground that the organism seen by Harz was so poorly described and illustrated that it is unrecognizable by present day standards, and that therefore a new name could well be applied to the organism of actinomycosis in cattle; that further the name *Actinomyces* was the first one applied to cultivated, aerobic, spore-forming species which can be recognized by present day standards. This has been the attitude of a number of recent medical mycologists, especially the Italian workers (Ciferri and Redaelli, 1929; Baldacci, 1939).

Critics of Waksman's classification have, however, maintained that while Harz' description of his organism is perhaps vague, there is no question concerning the nature of the disease he studied, and that the chances are overwhelmingly in favor of his having actually observed the anaerobic pathogenic filamentous organism first described by Israel. Further, under the Botanical Code, the name *Actinomyces* must be applied either to the organism of "lumpy jaw" or not used at all.

This problem became particularly significant in view of the need for the revision of the *Actinomycetales* for the sixth edition of Bergey's Manual which is being undertaken by the authors of this paper. We have been reluctant to deviate from Waksman's classification because we have felt that to abandon the name *Actinomyces* for the large group of aerobic spore-forming species would cause more confusion than to adopt the name *Cohnistreptothrix*, already in wide usage, for the anaerobic, pathogenic species. It appears, however, that under the code we have no such choice open to us. We believe that, to avoid similar dilemmas in the future, the code should be modified to permit generic names in wide usage based upon *type cultures* to have priority over names based merely upon morphologic descriptions.

¹ The authors wish to express their sincere appreciation to Dr. R. E. Buchanan for his ever-ready suggestions and criticisms concerning many of the difficult problems arising from the classification of this important group of microorganisms.

² The Editor desires to record with the deepest regret the death of Dr. Henrici some ten days after this paper was submitted.

It is our desire in this paper to present a classification of these organisms which will retain the morphological subdivisions presented by Waksman (1940), as seems to be considered desirable by most workers in this field, but to apply to these subdivisions names which will be acceptable under the Microbiological Code, and which we hope will become permanent, thus ending the intolerable confusion now existing.

If the name *Actinomyces* is to be restricted to the anaerobic, pathogenic species, a new generic name must be found for the aerobic, saprophytic spore-forming species. We have carefully reconsidered all of the names previously applied to organisms of this type. It is not necessary here to discuss the non-validity of such names as *Streptothrix*, *Cladothrix*, *Actinocladothrix*, *Discomyces*, *Oospora*, etc. which must be rejected either because they were first applied as synonyms of *Actinomyces* or were previously applied to entirely different sorts of organisms. The name *Nocardia*, however, requires special consideration.

This name, introduced by Trevisan in 1888, has been widely applied, sometimes to all of the actinomycetes, sometimes to the saprophytic aerobic species only. Breed and Conn (1919) clearly state the status of this name as follows:

In the original paper, as in the paper by DeToni and Trevisan, five species are given in the genus, the first of these being *N. farcinica* Trevisan, the species described, but not named, by Nocard (1888). While this species is not definitely named as the type species, there is not the slightest question but that Trevisan regarded it as the type species of the new genus. *N. actinomyces* Trevisan (Syn. *Actinomyces bovis* Harz) is given as the second species followed by *N. foersteri* (Cohn) Trevisan (Syn. *Streptothrix foersteri* Cohn).

The species *N. farcinica* must therefore stand as the type species if the term *Nocardia* is used no matter what limits are set for the genus.

Breed and Conn further concluded that:

There appears to be no justification for the use of the term *Nocardia* Trevisan for the entire group of organisms included in the Actinomycetaceae. It may however be properly used for a subdivision of the genus *Actinomyces*, provided however *N. farcinica* is retained in the genus *Nocardia* and is established as the type of the genus.

Now just such a situation has arisen. It has become increasingly apparent through the work of several people, but especially that of Ørskov (1923) and Jensen (1931), that the organisms which have been grouped together as actinomycetes can be rather sharply subdivided into a group which multiplies by fragmentation of the mycelium into oidia and a group which multiplies by the production of spores in aerial hyphae. To the former group Jensen gave the generic name of *Proactinomyces*.

If, however, the position of Breed and Conn is sound (and it seems so to us), the name *Nocardia* must have precedence over *Proactinomyces*, for there is no doubt that the species described by Nocard is a typical *Proactinomyces* in the sense of Jensen.

In addition to Nocard's original description, the characteristics of the organism which he isolated from "farcin du boeuf" have been studied from authentic subcultures by Musgrave, Clegg and Polk (1908), Ørskov (1923), and Lieske (1921), all of whose descriptions are in essential agreement. The multiplication

by fragmentation of the mycelium and the lack of aerial hyphae-forming spores is clear in nearly all of these descriptions. Acidfastness, a character common to a number of species of the genus *Proactinomyces* Jensen, was described by Feistmantel (1902). Apparently subcultures of this organism were distributed for some time from the Pasteur Institute and from the Kral collection, and authentic cultures are possibly still extant, though it is questionable whether the culture listed as *Actinomyces farcinicus* in the American Type Culture Collection is this species.

We conclude therefore that *Nocardia* Trevisan is the valid name for the aerobic, fragmenting non-sporulating types of actinomycetes, having priority over *Proactinomyces* Jensen.

There remains the consideration of valid names for the aerobic sporulating species. We have not found in previous literature any valid generic name which can be applied to those species which produce spores in chains, spores that are apparently formed within the hyphae, and which are characteristic of the large group of saprophytic soil actinomycetes. It is true that Wollenweber (1921) proposed to divide the genus *Actinomyces* into two subgenera, *Pionnothrix* lacking aerial mycelium, and *Aerothrix* with aerial mycelium, and it might be argued that the latter is a valid generic name for the spore-forming species. But the species listed under these two subgenera indicate that Wollenweber missed the essential differential character, which is not the production of aerial hyphae, but the production of spores. After surveying this situation we have with reluctance concluded that the only solution for this problem is to coin a new generic name for the aerobic, saprophytic actinomycetes which form catenulate spores, and we propose the name *Streptomyces*, which, so far as we can learn has never been used before and which indicates the essential character of the group. Since the Botanical Code recommends that family names be derived from generic names, the new family name for the spore-forming actinomycetes is proposed as *Streptomycetaceae*.

Family Streptomycetaceae. Actinomycetes with branched slender mycelium, rarely or not septate, forming spores on aerial hyphae, not fragmenting into oidia. There are two genera, *Streptomyces* and *Micromonospora*.

Genus Streptomyces. *Streptomycetaceae* forming spores in chains on aerial hyphae. Spores are apparently endogenous in origin, formed by a segregation of protoplasm within the hypha into a series of round, oval or cylindric bodies. Chains of spores are often spirally coiled. Sporophores may be simple or branched.

We have selected as the type species of this newly-named genus, *Streptomyces albus* (Rossi-Doria emend Krainsky) comb. nov. This species was formerly known as *Actinomyces albus* Krainsky and first described as *Streptothrix alba* Rossi-Doria. This is one of the commonest and best known species of the group, and while it may later be subdivided into further species, it is at present as definite as any others. It has been recently studied intensively by Duché (1934) and by Baldacci (1939). It is colorless with white aerial mycelium, forming ovoidal spores in coiled chains on lateral branches of the aerial hyphae. It is

proteolytic, liquefying gelatin and peptonizing milk with the production of an alkaline reaction in the latter. It does not produce any soluble pigment either on an organic or synthetic medium, but does produce a characteristic earthy or musty odor.

The name *Micromonospora* Ørskov, applied to those forms which produce single conidia on lateral branches, is apparently still valid. It is true that Tsiklinsky (1899) had previously applied the name *Thermoactinomyces* to species of this group, whose identity is clear from photomicrographs. But in her description of the genus she also included thermophilic species with catenulate spores, basing the genus on temperature relations rather on morphology.

Finally we wish to remark on the proposal by Stanier and van Niel (1941) that the genus *Mycobacterium* be transferred from the *Actinomycetales* to the *Eubacteriales*. We believe that those who have extensively studied microorganisms within this group, especially the pathogenic actinomycetes, will agree that such a separation is too violent and unnatural. One frequently finds strains which require much careful study before it can be decided whether they are to be considered as mycobacteria or actinomycetes. Strains may form mycelium for only a few hours, then fragment into bacillary forms; or may form mycelium for a few transfers when freshly isolated, and appear forever afterwards only as acidfast rods. There are also known organisms transitional between the actinomycetes and the staphylococci. It is possible that Badian's cytological studies may eventually show a relationship between the endospores of the *Bacillaceae* and those of the group we have designated as *Streptomyces*. All of these considerations lead us to believe that a more natural system would be arrived at by transferring the gram-positive bacteria from the *Eubacteriales* to the *Actinomycetales* than to transfer *Mycobacterium* to the *Eubacteriales*. Present knowledge however does not justify so drastic a change, but we prefer to retain the acidfast bacteria in the *Actinomycetales*. We do agree with Stanier and van Niel that the gram-negative genus *Mycoplasma* should not be retained within this order. We also agree with Stanier and van Niel as well as with Breed that the genus *Corynebacterium* be transferred to the *Lactobacteriaceae*.

In view of the above considerations we propose the following nomenclature and classification of the *Actinomycetales* which we hope will satisfy both the Microbiological and Botanical Codes and the taxonomic data:

- A. Mycelium rudimentary or absent. Family *Mycobacteriaceae* Chester
 - I. Acidfast organisms. *Mycobacterium* Lehmann and Neumann
- B. True mycelium produced.
 - I. Vegetative mycelium fragments into bacillary or coccoid elements. Family *Actinomycetaceae* Buchanan.
 - a. Anaerobic or microaerophilic, parasitic, not acidfast. *Actinomyces* Harz.
 - b. Aerobic, partially acidfast or non-acidfast. *Nocardia* Trevisan.
 - II. Vegetative mycelium not fragmenting into bacillary or coccoid elements. Family *Streptomycetaceae* Waksman and Henrici.
 - a. Multiplication by conidia in chains from aerial hyphae. *Streptomyces* Waksman and Henrici.
 - b. Multiplication by single terminal spores on short sporophores. *Micromonospora* Ørskov.

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THE USE OF SODIUM AZIDE (NaN_3) AND CRYSTAL VIOLET IN A SELECTIVE MEDIUM FOR STREPTOCOCCI AND *ERYSIPELOTHRIX RHUSIOPATHIAE*¹

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The isolation and study of certain microorganisms are often very difficult because of the presence of other organisms which overgrow the desired one on ordinary culture media. This is especially true when pathogens are to be isolated from contaminated material such as intestinal contents or from the upper respiratory tract where there is an abundance of saprophytic organisms. Contaminated milk samples mailed to the laboratory for mastitis diagnosis are usually overgrown to such an extent that pure cultures of the streptococci present can not be obtained for identification except with difficulty. It was in this connection that a study of selective media for streptococci was begun.

Edwards (1933, 1938) described two selective media which were of value. The first was a crystal-violet esculin blood agar and the other a crystal-violet sodium-azide broth. Hartmann (1936) investigated the value of several substances for the selective cultivation of mastitis streptococci from contaminated material. Of the substances tested, sodium azide appeared to be most efficient. Snyder and Lichstein (1940) used sodium azide as an inhibitor of gram-negative bacteria.

A combination medium employing sodium azide and crystal violet has been developed and found to have some advantages over the media referred to above. A description of this medium and its uses follows.

METHODS

The base medium used in the investigation was either tryptose broth or tryptose agar. Broth medium was prepared by dissolving 3 grams of beef extract, 15 grams of bacto-tryptose, and 5 grams of NaCl in 1000 ml. of distilled water. The pH was determined by means of an electrometer and was adjusted to 0.2 of a pH unit above the final pH desired. The medium was filtered through absorbent cotton and dispensed in 150 ml. quantities into 200 ml. Erlenmeyer flasks. These were sterilized by autoclaving 20 minutes at 15 lbs. pressure. Tryptose agar base was prepared by the addition of 18 grams of agar per liter to the broth base.

A stock solution of crystal violet was made by adding 0.25 gram of crystal violet powder, dye content 94 per cent, to 100 ml. of distilled water. Sodium

¹ A thesis presented in partial fulfillment for the Master of Science Degree at Iowa State College.

azide² stock solution was prepared by dissolving 1 gram of sodium azide powder in 25 ml. of distilled water. These stock solutions were sterilized by autoclaving 20 minutes at 15 lbs. pressure. When gentian violet was used, a stock solution was prepared in the same manner as for crystal violet. Gentian violet employed in ordinary staining methods was used. Various concentrations of the inhibiting substances were made by adding appropriate amounts of the stock solutions to the sterile base media with sterile pipettes. Glucose was added in some cases as an enrichment substance. This was added before sterilization in amounts to make 1 per cent concentration. When blood agar was used it was prepared by adding sterile, citrated horse blood to the melted agar base which was cooled to 48°C. The horse blood contained 0.5 per cent sodium citrate to prevent coagulation and was stored at 5°C. for 1 to 30 days before use. There was little or no hemolysis of the blood up to 30 days storage. After the various substances were added to the medium it was dispensed into sterile culture tubes in approximately 5 ml. amounts. Tubes containing agar were placed in a slanting position until hardened. The final pH of the medium was determined at the end of the pouring operation. The tubes of poured media were incubated overnight to determine sterility and were inoculated on the following day.

The stock cultures of the test organisms were grown on tryptose agar pH 7.4. Before inoculation into the test media, transfers were made to tryptose broth pH 7.4 and incubated 18–24 hours at 37°C. These 18–24 hour broth cultures were diluted in sterile saline immediately before inoculation into the test media. Due to great variation in the amount of growth of the different organisms at 18–24 hours incubation, it was necessary to dilute those organisms which grew abundantly considerably more than those which grew more slowly. Two drops of the 18–24 hour broth cultures of *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella choleraesuis*, *Pasteurella multocida*, *Eberthella typhosa*, *Proteus vulgaris*, *Bacillus subtilis* and *Staphylococcus aureus* were diluted in 10 ml. of sterile saline. Streptococci and micrococci were diluted 5 drops in 10 ml. of saline. *Erysipelothrix rhusiopathiae*, *Corynebacterium pyogenes*, *Brucella abortus* and *Listerella monocytogenes* were diluted 10 drops in 10 ml. of saline. The test media containing the various dilutions of sodium azide and crystal violet were inoculated with 1 drop of the diluted saline suspensions delivered from a 1 ml. pipette. Plate counts of 1 drop of the saline suspensions showed that the numbers of organisms inoculated usually varied between 2,000 and 10,000 organisms. The inoculated cultures were incubated at 37°C. Observations were made at 24, and 48 hours, and final results recorded after 72 hours incubation.

RESULTS

The presence of growth was usually determined macroscopically but in doubtful cases microscopic examinations were made. The amount of growth in each case was determined by comparing the growth with that of a control not containing inhibitory substances. Thus, the control is always designated as +++

² Obtained from Fairmount Chemical Company, Newark, New Jersey. Manufacturers analysis 95% sodium azide.

(see table 1) so that the comparison is a measure of the inhibitory action rather than a comparison of the amounts of growth of one organism contrasted to another. Growths designated +++ are comparable to that of the controls, ++ and + growths designate varying degrees of inhibition of growth, ± indicates faint growth, often 1 colony, 0 indicates no growth or complete inhibition. Many instances of ± growth occurred only after 72 hours incubation.

In table 1 the inhibitory effect of sodium azide blood agar upon the growth of the various organisms is shown. The streptococci, except *S. lactis*, are not

TABLE 1

Inhibitory action of various concentrations of sodium azide in blood agar

	CONCENTRATION OF SODIUM AZIDE					
	1-500	1-1,000	1-2,000	1-3,000	1-5,000	Control
	Tryptose agar and 5 per cent horse blood. Final pH 6.8					
<i>Streptococcus pyogenes</i> A	++	++	+++	+++	+++	+++
<i>S. pyogenes</i> C.....	++	+++	+++	+++	+++	+++
<i>S. agalactiae</i>	++	++	+++	+++	+++	+++
<i>S. dysgalactiae</i>	++	++	+++	+++	+++	+++
<i>S. uberis</i>	+++	+++	+++	+++	+++	+++
<i>S. viridans</i>	++	+++	+++	+++	+++	+++
<i>Diplococcus pneumoniae</i>	+++	+++	+++	+++	+++	+++
<i>S. lactis</i>	0	±	++	++	+++	+++
<i>Micrococcus</i> Sp.	+	++	+++	+++	+++	+++
<i>Staphylococcus aureus</i>	0	±	+++	+++	+++	+++
<i>E. rhusiopathiae</i>	++	++	+++	+++	+++	+++
<i>P. aeruginosa</i>	0	0	±*	±	+++	+++
<i>S. choleraesuis</i>	0	0	0	±	+++	+++
<i>Proteus vulgaris</i>	0	0	0	±	+++	+++
<i>E. coli</i>	0	0	0	±	±	+++
<i>E. typhosa</i>	0	0	0	±	±	+++
<i>Aerobacter aerogenes</i>	0	0	0	±	±	+++
<i>P. multocida</i>	0	0	0	+	+	+++
<i>L. monocytogenes</i>	0	0	0	±	+	+++
<i>C. pyogenes</i>	0	0	0	±	+	+++
<i>B. subtilis</i>	0	0	0	±	+	+++
<i>Brucella abortus</i>	0	0	0	±	+	+++

* One colony after 72 hours incubation.

inhibited in any of the concentrations of sodium azide in blood agar. *Diplococcus pneumoniae* likewise is not inhibited. *E. rhusiopathiae* is only slightly inhibited. The micrococci, *Staphylococcus aureus* and *Streptococcus lactis* are definitely inhibited by the concentrations above 1-2,000. In the case of *S. aureus*, a marked inhibition was noted in the 1-500 and 1-1,000 concentrations whereas no inhibition was observed in the 1-2,000 concentration. The other species of organisms are definitely inhibited in concentrations greater than 1-5,000. *P. aeruginosa* proves to be more resistant than any other of the gram-negative organisms.

A series of tests were made using the same concentrations of sodium azide as in table 1, except that tryptose broth was used instead of tryptose blood agar. Results of these tests are similar to those obtained on blood agar. Several of the gram-negative rods, *P. aeruginosa*, *E. coli*, *S. choleraesuis*, and *E. typhosa*, are not inhibited at 1-2,000 concentration as is the case in the blood agar media containing 1-2,000 sodium azide. More inhibition of the streptococci was noted in the broth of 1-500 concentration than on blood agar of the same concentration of sodium azide.

The action of sodium azide in broth at pH 7.4 instead of pH 6.8 was compared. It was noted that there was considerably less inhibition at pH 7.4 than at pH 6.8. This is particularly true in the 1-500 concentration among the streptococci. For example, *S. lactis* is completely inhibited in the 1-500 and 1-1,000 concen-

TABLE 2

Inhibitory action of crystal violet alone and in combination with sodium azide

	TRYPTOSE AGAR pH 6.8				
	Crystal violet 1-200,000	Crystal violet 1-200,000, 5 per cent blood	Crystal violet 1-500,000, 5 per cent blood	Crystal violet 1-500,000, NaN ₃ 1-2000, 5 per cent blood	Crystal violet 1-100,000, NaN ₃ 1-1000, 5 per cent blood
<i>S. pyogenes</i> A.....	0	+	+++	+++	0
<i>S. pyogenes</i> C.....					
<i>S. agalactiae</i>					
<i>S. dysgalactiae</i>					
<i>S. uberis</i>	+	++	+++	+++	0
<i>S. viridans</i>					
<i>Micrococcus</i> sp.....					
<i>S. lactis</i>	0	0	±	±	0
<i>S. aureus</i>	0	0	0	0	0
<i>E. rhusiopathiae</i>	+++	+++	+++	+++	+++
Gram-negative rods.....	+++	+++	+++	0	0
<i>B. subtilis</i>	0	0	0	0	0
<i>C. pyogenes</i>					
<i>L. monocytogenes</i>					

trations at pH 6.8, while the same concentrations at pH 7.4 yield abundant growth. *S. aureus* is completely inhibited in the 1-500 concentration but abundant growth is obtained at pH 7.4 in all concentrations of sodium azide. *Brucella abortus* is completely inhibited by all concentrations of sodium azide at a pH of 6.8, while fair growth is obtained in all concentrations except 1-500 at a pH of 7.4.

Table 2 shows the inhibitory action of crystal violet on the various groups of organisms. The effect of the addition of 5 per cent blood is shown and the inhibitory action of various concentrations of crystal violet alone and in combination with sodium azide are compared. When the results were the same for different organisms they are grouped in order to make the material more concise.

It will be noted in table 2 that a concentration of 1-200,000 of crystal violet is inhibitory to the streptococci, staphylococci, micrococci, and the gram-positive

rods except *E. rhusiopathiae*. The gram-negative organisms grow abundantly in that concentration. The addition of 5 per cent blood to the medium decreases the effect of crystal violet and allows the growth of all streptococci except *S. lactis*. A concentration of 1-500,000 crystal violet allows the growth of all pathogenic streptococci, but inhibits *S. aureus* completely and permits only a few colonies of *S. lactis* to develop on the medium.

Addition of sodium azide to the medium containing crystal violet inhibits the growth of all of the species in the gram-negative-rod group and does not alter the effects of crystal violet.

It is possible to inhibit all organisms except *E. rhusiopathiae* completely by increasing the concentrations of crystal violet and sodium azide to 1-100,000 and 1-1,000 respectively.

It is further noted that colonies of *S. agalactiae* and *S. uberis* absorb enough crystal violet to give them a distinctly violet color. *S. viridans* and *S. dysgalactiae* absorb only a moderate amount and *S. pyogenes* none at all.

Gentian violet was used in a similar experiment and compared with crystal violet. The inhibitory action is practically the same as crystal violet except in minor instances.

During the course of this study it was noted that differences in hemolysis of blood agar varied to some extent with the streptococci, micrococci and pneumococci. *S. dysgalactiae* produced beta hemolysis in sodium azide blood agar as compared to gamma hemolysis in blood agar. A wide greenish zone of hemolysis around colonies of organisms commonly designated as alpha hemolytic was also observed. The zone of hemolysis of beta hemolytic streptococci, such as *S. pyogenes*, was definitely extended.

It is universally recognized that the addition of a fermentable carbohydrate to blood agar commonly reduces and often inhibits the production of hemolysin by streptococci. Since hemolysis is more marked in the presence of sodium azide, a series of tests were made in a medium to which one per cent glucose was added. It is noted that in the presence of glucose, species of hemolytic streptococci are unable to produce hemolysis while on media not containing glucose definite hemolysis is produced. In this study it was observed that alpha hemolytic streptococci produced a yellowish-green color which spread over the entire plate in the presence of glucose while that characteristic was not found in media not containing this carbohydrate.

Microscopic examination of stained smears from the inoculated media containing higher concentrations of sodium azide, in some cases revealed many unusual morphological characteristics. Several species, particularly the gram-negative rods, were so changed as to be unrecognizable. For example, *Aerobacter aerogenes* was observed to form large ovoid cells several times the length and width of those seen from ordinary media. Transfers of these atypical cells into media containing no sodium azide produced cells typical of the species in size and shape. The other species of gram-negative rods and *Bacillus subtilis* exhibited similar morphologic changes. The streptococci in general were not affected morphologically by sodium azide.

DISCUSSION

A selective medium using sodium azide and crystal violet as inhibiting agents is able to eliminate much of the difficulty in culturing streptococci and *E. rhusiopathiae* from contaminated materials. A concentration of 1–2,000 sodium azide in blood agar having a pH of 6.8 did not affect the growth of these organisms and is very effective in inhibiting gram-negative organisms. The pH of the medium is an important factor in obtaining the maximum effect of the sodium azide. Edwards (1938) found that *E. coli* was inhibited by a 1–10,000 concentration of sodium azide if the pH of the medium was 6.8 but was only partially inhibited when the pH was 7.4. The results of this study not only confirm Edward's observation on *E. coli*, but show a similar effect on all organisms which are inhibited by sodium azide.

The mechanism by which sodium azide is able to inhibit growth of certain bacteria was not studied. Since this substance is an inhibitor of the enzyme catalase, it is possible that its inhibitory effect may be due to the inhibition of certain enzyme systems within the bacterial cell. Schattenfroh (1896) who was the first to study the effects of azide compounds on bacteria found that both sodium and ammonium azides inhibited bacterial growth.

Micrococci, *S. aureus*, *D. pneumoniae*, and *E. rhusiopathiae* were not inhibited by sodium azide. The addition of 1–500,000 crystal violet to the medium increased the selectivity of the medium by inhibiting *S. aureus*. It was not possible to change the concentrations of the inhibitory substances to eliminate the other three species without also inhibiting the streptococci. However, the colonies of micrococci can usually be distinguished from streptococci by their larger size and greenish zone of hemolysis around them when grown on sodium azide blood agar. In cases when *E. rhusiopathiae* or *D. pneumoniae* may occur with streptococci, they must be differentiated by other recognized procedures.

It is noted that *E. rhusiopathiae* is the most tolerant of all species studied being able to grow in sodium azide 1–1,000 and crystal violet 1–100,000 concentrations. This fact makes it possible to increase the concentrations to a point where only this organism and no other will grow. A crystal-violet sodium-azide blood agar containing 1–1,000 sodium azide and 1–100,000 crystal violet was prepared. Saline suspensions of all the species of organism used in this study were mixed and cultured on this medium. Pure cultures of *E. rhusiopathiae* were obtained from the mixture on this medium.

The effect of sodium azide on hemolysis of red blood cells is of interest. All hemolytic action appears to be enhanced. Possibly the methemoglobin formed by the combination of sodium azide and hemoglobin is more easily altered than oxyhemoglobin. Certain organisms may be capable of changing methemoglobin and not oxyhemoglobin because some streptococci were able to produce hemolysis on sodium azide blood agar but were non-hemolytic on ordinary blood agar. The marked discoloration of sodium azide blood agar by beta hemolytic strains of streptococci described by Snyder and Lichstein (1940) was not confirmed. In fact, beta hemolytic strains of streptococci used in this study produced a wide,

clear zone of hemolysis on sodium azide blood agar which was wider than that seen on ordinary blood agar.

CONCLUSIONS

1. A concentration of 1-2,000 sodium azide in 5 per cent blood agar having a pH of 6.8 was found to be most effective in inhibiting organisms other than the streptococci.

2. Sodium azide in a medium at pH 6.8 is a more effective inhibiting agent than when used in a medium at pH 7.4.

3. A concentration of 1-500,000 crystal violet appears to be most effective in inhibiting organisms not inhibited by sodium azide.

4. Pathogenic streptococci grew well in 1-500,000 crystal violet while *Staphylococcus aureus* did not grow in this concentration of the dye.

5. A combination of 1-2,000 sodium azide and 1-500,000 crystal violet in 5 per cent blood agar having a pH of 6.8 was found to inhibit almost all organisms except the streptococci.

6. *Diplococcus pneumoniae*, *Erysipelothrix rhusiopathiae* and the micrococci were not effectively inhibited on the combination medium.

7. Hemolytic properties of the streptococci grown on sodium azide blood agar are somewhat different from those observed on ordinary blood agar.

8. *E. rhusiopathiae* is not inhibited by concentrations of crystal violet and sodium azide which inhibit all other organisms studied including the streptococci.

9. A blood agar medium having a pH of 6.8 and containing 1-1,000 sodium azide, 1-100,000 crystal violet and 5 per cent blood is recommended for the selective isolation of *E. rhusiopathiae* from contaminated material.

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DECOMPOSITION OF VITAMIN C BY BACTERIA¹

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The decomposition of vitamin C by bacteria is of double interest to the bacteriologist. Preliminary *in vitro* studies by Young and James (1942) showed that enteric bacteria of the colon-aerogenes group destroy ascorbic acid with great facility; these findings suggest that, in spite of a diet adequate in this substance, symptoms of vitamin C deficiency in some persons may be traced to an intestinal flora high in ascorbic-acid-fermenting bacteria. Einhauser (1936), Hetenyi (1935) and Marin (1936) offer the suggestion that the failure of response to a diet rich in vitamin C, and the positive effects of intramuscular and intravenous injections of this substance, indicate bacterial destruction of ascorbic acid in the gastro-intestinal tract before absorption can take place.

The extreme importance of vitamin C in human physiology and the significance of loss of this substance to the body, regardless of the manner of destruction, are becoming increasingly evident as the result of war research and conditions arising naturally or unavoidably among the fighting forces. Holmes (1942) has indicated that vitamin C may be destroyed by infection, by a number of industrial poisons of a military nature, especially materials used in the manufacture of T.N.T., and that appreciable quantities are lost in heavy perspiration. He reviews the beneficial effects of vitamin C therapy in various types of shock, including heat, traumatic, post-operative and allergic shock. Vitamin C appears to play a significant role in the healing of fractures and of wounds in general. Results reported by Holmes indicate that administration of the vitamin is very effective in the treatment of poisoning due to benzene, toluene, lead, zinc, and basic materials used in the manufacture of T.N.T. and other explosives. Detoxification followed adequate therapy, and there was a repairing action of the poisoned blood formation centers and other affected tissue. Thus, it appears that in numerous common pathological conditions the function of vitamin C extends far beyond the role which it plays in normal metabolism, and that the amount required under these conditions greatly exceeds that needed daily by normal human adults, which according to King and Menten (1935) is only 5 milligrams.

Apart from the influence on human nutrition, the relation of microbic destruction of ascorbic acid to bacterial metabolism is also of considerable interest. Kendall and Chinn (1938) suggested that the vitamin is used by some bacterial species as a carbon food source. The results of preliminary work by Young and James (1942) indicate that the vitamin is utilized in growth as an energy source by certain enteric bacteria. The decomposition was found to take place under

¹ This paper covers in part the dissertation submitted to the Graduate School of Yale University by the senior author as part requirement for the degree of Doctor of Philosophy.

anaerobic as well as aerobic conditions; and, since cell-free filtrates had no effect on the substance, the activation was believed to be associated with the endoenzyme system of the living bacterial cell. The rate of decomposition was found to be most rapid when cells were most viable, pointing to involvement of the respiratory mechanism of the bacterial cell. The presence in the medium of an excess of fermentable substance like glucose delayed or prevented the decomposition, suggesting that in the absence of a readily available carbohydrate, like glucose, the vitamin is used as a substitute for the same purpose.

Because of the implied bearing of vitamin C destruction by bacteria on human nutrition, and because of the importance of the interrelationship between bacterial decomposition of ascorbic acid and bacterial metabolism, the present research was undertaken, with the following objectives in mind:

Making a general survey of the extent of the vitamin-C-decomposing ability of members of various genera in the order *Eubacteriales*.

Determination of the influence of various carbohydrates on the decomposition.

Study of the relation between bacterial growth and the stability of ascorbic acid in synthetic and nutrient broth mediums.

An inquiry into the mode of decomposition of vitamin C by bacteria.

METHODS EMPLOYED

Ascorbic acid nutrient broth was employed in determining the vitamin-C-decomposing ability of various bacterial species. The broth was prepared as follows: a weighed amount of crystalline ascorbic acid was dissolved in a small amount of water, filtered through a Berkefeld filter and the pH adjusted to about 7.0 with a concentrated sterile solution of K_2HPO_4 . The filtrate was added to sterile nutrient broth, giving a final concentration of 0.50–0.55 mg. per ml. of broth.

When carbohydrates were used in the medium they were present in a final concentration of 1 per cent. In some of the studies, synthetic ascorbic acid broth and other modifications of the culture medium were employed; these are described later.

After removing an aliquot portion for immediate analysis, the ascorbic acid nutrient broth was distributed in 20 ml. portions in sterile 7 x $\frac{7}{8}$ inch colorimeter tubes, which were then inoculated with the various organisms studied. All cultures and an uninoculated control were incubated under anaerobic conditions at 37°C. Titrations were made frequently, using a modified procedure of Tillman's sodium 2,6-dichlorophenolindophenol method adapted to the Evelyn photoelectric colorimeter. Duplicate portions of 1 ml. of a 1/10 dilution of the culture and of the control were added to 4 ml. of meta-phosphoric-acid buffer solution,² thus providing an optimum H-ion concentration of approximately pH 3.5 for the test

² *Preparation of meta-phosphoric-acid buffer solution.* A stock solution of 6% meta-phosphoric acid was prepared from sticks of glacial metaphosphoric acid. The working reagent consisted of a 3% solution of the acid adjusted to pH 3.5 with citric acid-NaOH buffer solution, as described by Bessey (1938). The buffer was prepared by adding 210 gm. of citric acid to 2,000 ml. of NaOH, and increasing the volume to 2,500 ml. with distilled water. It was found that about 25 ml. buffer were required to adjust 100 ml. of the 3% HPO_3 to pH 3.5.

medium. Then 5 ml. of the indophenol dye solution³ were added and the color intensity measured with the photoelectric colorimeter. By noting the units of galvanometer deflection and referring the reading to that on a standard reference curve plotted against increasing acid-buffer dilutions of a carefully weighed amount of ascorbic acid, the concentration of vitamin C in the test samples was determined. Under the experimental conditions employed the reaction was immediate; the end point was stable, and the readings could be taken rapidly.

The photoelectric colorimeter was also used to detect growth increase. The effect of the vitamin upon growth was noted by comparing the galvanometer deflection due to turbidity resulting from bacterial growth in ascorbic acid medium with that occurring in broth lacking the vitamin. Since colorimeter tubes were used for all of the cultures, the culture tubes were merely placed in the apparatus after thorough shaking to insure uniform turbidity, and the galvanometer deflection readings referred to standard growth curves in which deflection values were plotted against plate counts of various bacterial species studied.

The anaerobic procedure employed in this investigation consisted in evacuating anaerobic jars and replacing the air with about 10 per cent carbon dioxide and 85-90 per cent hydrogen gas. The culture tubes were placed in the jars, together with a small amount of heated palladinized asbestos, which served as catalyst to effect combination of residual oxygen with hydrogen in the evacuated jar. Anaerobic conditions were employed for the following reasons: (1) because of the importance attached to gastro-intestinal destruction of vitamin C, where the conditions would be anaerobic; (2) because auto-oxidation of vitamin C is retarded greatly under anaerobic conditions; and (3) because, if the decomposition is carried out in the absence of molecular oxygen, implying the operation of a dehydrogenase system, some information is given as to the nature of the decomposition process by bacteria.

GENERAL SURVEY OF THE ABILITY OF VARIOUS SPECIES OF BACTERIA TO DECOMPOSE VITAMIN C

Perhaps the most outstanding observation is the frequency with which the vitamin-C-decomposing property occurs among members of the enteric group of bacteria. As shown in table 1, members of the following genera oxidize ascorbic acid: *Escherichia*, *Aerobacter*, *Salmonella*, *Eberthella*, *Streptococcus* (enterococci), *Streptococcus* (hemolytic), *Encapsulatus*, *Vibrio*. Barring two exceptions, it was observed that when one species in a genus possessed this property, it was common to all other genus members tested. *Proteus morgani* actively destroyed ascorbic acid, whereas other species of *Proteus* lacked this ability; however, in many of its characteristics *Proteus morgani* appears to be related more to

³ *Preparation of indophenol-dye solution.* About 0.1 gm. of the dye powder, sodium 2,6-dichloro-benzenoneindophenol, was added to a liter of warm distilled water, mixed well and filtered. The concentration of dye was so adjusted by adding more water that when 5 ml. of the solution were added to 5 ml. of meta-phosphoric-acid-buffer a galvanometer deflection of exactly 80 units was obtained. Increasing amounts of ascorbic acid decrease the dye color intensity and so decrease the galvanometer deflection. The solution was kept in the refrigerator and remained stable several days.

TABLE 1
Decomposition of ascorbic acid by various bacteria

ORGANISMS INCUBATED UNDER ANAEROBIC CONDITIONS AT 37°C. IN ASCORBIC ACID NUTRIENT BROTH BUFFERED AT pH 7.0	STRAINS OR SPECIES	ASCORBIC ACID OXIDATION*	GAS PROD.†	RECOVERY WITH H ₂ S‡	REDUCTION OF NILE BLUE§
<i>Escherichia coli</i>	28 strains	+	+	—	+
<i>Aerobacter aerogenes</i>	12	+	+	—	+
<i>Aerobacter cloacae</i>	1	+	+	—	+
<i>Strep. liquefaciens</i>	2	+	—	—	+
<i>Strep. zymogenes</i>	2	+	—	—	
<i>Strep. agalactiae</i>	2	+	—	—	
<i>Strep. fecalis</i>	15	+	—	—	+
<i>Strep. pyogenes</i>	1	+	—	—	
<i>Encapsulatus pneumoniae</i>	1	+	+	—	
<i>Vibrio cholerae</i> (at pH 7.8).....	1	+	—	—	
<i>Vibrio metchnikovi</i> (pH 7.8).....	1	+	—	—	
<i>Eberthella typhi</i>	1	+	—	—	
<i>Proteus morgani</i>	2	+	+	—	+
<i>Salmonella paratyphi</i>	2	+	+	—	
<i>Salmonella schottmülleri</i>	2	+	+	—	
<i>Salmonella enteritidis</i>	2	+	+	—	+
<i>Salmonella aertrycke</i>	3	+	+	—	+
<i>Salmonella suispestifer</i>	2	+	+	—	
<i>Salmonella anatum</i>	1	+	+	—	+
<i>Salmonella gallinarum</i>	10	+	—	—	+
<i>Salmonella pullorum</i>	33	—			+
<i>Proteus vulgaris</i>	3	—			+
<i>Shigella dysenteriae</i>	Flexner	—			
<i>Shigella dysenteriae</i>	Shiga	—			
<i>Staphylococcus aureus</i>	2	—			
<i>Staphylococcus albus</i>	1	—			
<i>Brucella</i>	3 species	—			
<i>Pasteurella leptiseptica</i>	1	—			
<i>Serratia marcescens</i>	4	—			+
<i>Pseudomonas aeruginosa</i>	2	—			
<i>Flavobacterium vitaminosus</i>	1	—			
<i>Alcaligenes viscosum</i>	2	—			—
<i>Alcaligenes fecalis</i>	2	—			—
Incubated under aerobic conditions.....					
<i>Pseudomonas fluorescens</i>	2	—			
<i>Bacillus</i>	7 species	—			+

+, complete decomposition in 20–48 hours, as shown by periodic quantitative analysis. Initial amount 0.55 mg. per ml. —, no action after 7–10 days.

* Growth of all cultures listed was moderate to luxuriant in ascorbic acid nutrient broth.

† +, production of gas. Quantitative tests showed a positive correlation between increase in gas production and decrease in ascorbic acid content of the medium. —, no gas production.

‡ —, no recovery of ascorbic acid, and hence irreversible oxidation.

§ +, complete reduction of Nile blue under anaerobic conditions by ascorbic acid substrate in the presence of "resting cells." —, no reduction.

the colon-aerogenes group and the salmonellas than to the *Proteus* genus. *Salmonella pullorum* alone, of the various species of *Salmonella* tested failed to de-

compose ascorbic acid; but this organism is relatively asaccharolytic as compared with other members of the group.

Furthermore, with only a few exceptions, not indicated in table 1, it was found that when a given strain fermented the vitamin all strains of the same species also destroyed it. The exceptions were invariably variant strains which showed atypical reactions in other biochemical tests, or failed to grow in the ascorbic acid broth.

Aerobic spore-formers, a few intestinal and many non-intestinal forms did not attack the vitamin in nutrient broth, even though good growth was evident. Members of the following genera failed to act on the substance: *Bacillus*, *Alcaligenes*, *Pseudomonas*, *Proteus*, *Shigella*, *Staphylococcus*, *Brucella*, *Pasteurella*, *Serratia*, *Flavobacterium*, *Chromobacter*, *Achromobacter*.

Vitamin-C-decomposing bacteria appear to possess initial capacity to destroy ascorbic acid, no training or acclimatization technique being necessary. Another outstanding observation is the "all or none" character of the reaction. In every instance where an organism attacked the vitamin at all the decomposition was complete; that is, all of the vitamin in the test medium was destroyed or utilized. With most cultures the destruction was rapid, occurring within 12-22 hours.

Some organisms, especially members of the *Serratia*, *Flavobacterium* and *Pseudomonas* genera, showed marked acceleration of growth in the presence of vitamin C in nutrient broth; yet, these organisms were unable to attack or decompose the substance. The "growth factor" effect may have been due to a favorable influence of a lowered potential of the medium, ascorbic acid being a strong reducing substance.

INFLUENCE OF VARIOUS CARBOHYDRATES ON THE DECOMPOSITION

In earlier studies it was observed that the presence of either glucose or lactose in ascorbic acid nutrient broth deflected attack upon the vitamin by members of the colon-aerogenes group under both aerobic and anaerobic conditions. When the concentration of glucose or lactose was varied, the vitamin disappeared rapidly from the medium, following complete utilization of these sugars.

The present investigation sought to determine the extent to which the vitamin-sparing action occurs among the various groups of organisms that ferment the substance; and to correlate the sparing effect of different carbohydrates with the ability of the bacteria to ferment them. The list of carbohydrates included glucose, lactose, xylose, sucrose, dextrin and mannitol. They were added in 1 per cent concentration to 1 per cent peptone broth containing 0.50 mg. ascorbic acid per ml. buffered at pH 7.0. Several bacterial species listed in table 2 were selected from the large group of vitamin-C-decomposing bacteria to serve as control organisms. Since the principles underlying the results obtained with glucose and lactose apply also to those obtained with xylose, sucrose, dextrin and mannitol, the data for glucose and lactose broth only are presented in table 2.

From the results obtained it may be postulated that when an excess amount of carbohydrate is fermented rapidly by vitamin-C-decomposing bacteria, with acid or acid and gas production, ascorbic acid, when added to the same medium, will

as a rule be spared from oxidation. When the carbohydrate was not fermented at all or attacked slowly by a given species the vitamin was rapidly decomposed, without exception. As indicated in table 2, one strain of *Aerobacter aerogenes* fermented lactose rapidly, protecting the vitamin completely, whereas two other strains of the same species were slow lactose fermenters, and attacked ascorbic acid more readily, completely destroying the substance. Since all of the *Salmon-*

TABLE 2
Influence of glucose and lactose on the bacterial decomposition of vitamin C

ORGANISMS INCUBATED UNDER ANAEROBIC CONDITIONS AT 37°C. IN BROTH BUFFERED AT pH 7.0	GLUCOSE BROTH (1 PER CENT)			LACTOSE BROTH (1 PER CENT)				
	Plus vitamin C		Ferm. tube	Plus vitamin C		Ferm. tube		
	42 hours	168 hours		42 hours	168 hours	18 hours	30 hours	78 hours
<i>E. coli</i> (Ce).....	0.42*	0.33	AG†	0.46*	0.46	AG†		
<i>E. coli</i> (Dd).....	0.38	0.33	AG	0.47	0.47	AG		
<i>E. coli</i> (Co-3).....	0.47	0.44	AG	0.46	0.45	A	AG	
<i>A. aerogenes</i> (5) . . .	0.47	0.45	AG	0.47	0.43	AG		
<i>A. aerogenes</i> (11)....	0.40	0.25	AG	0.00		0	0	AG
<i>A. aerogenes</i> (14)....	0.47	0.35	AG	0.00		0	0	AG
<i>Strep. fecalis</i> (K).....	0.42	0.38	A	0.39	0.32	A	A	A
<i>Strep. fecalis</i> (U).....	0.42	0.35	A	0.40	0.30	A	A	A
<i>Strep. fecalis</i> (P)....	0.42	0.37	A	0.43	0.38	A	A	A
<i>Strep. liquefaciens</i>	0.41	0.37	A	0.00		0	A	A
<i>Sal. para B</i> (18).....	0.42	0.37	AG	0.00		0	0	0
<i>Sal. para B</i> (19) . . .	0.42	0.40	AG	0.00		0	0	0
<i>Sal. enteritidis</i>	0.42	0.41	AG	0.00		0	0	0
<i>Sal. aertrycke</i>	0.42	0.38	AG	0.00		0	0	0
<i>Sal. anatum</i>	0.42	0.39	AG	0.00		0	0	0
<i>Sal. gallinarum</i>	0.42	0.26	A	0.00		0	0	0
<i>Proteus morgani</i>	0.43	0.43	AG	0.00		0	0	0
Uninoculated control.....	0.33	0.28		0.33	0.28			

* Milligrams ascorbic acid per ml. broth. Initial amount: 0.50 mg. per ml.

† AG indicates both acid and gas production in ordinary glucose and lactose fermentation broths containing no ascorbic acid. 0 indicates no action on the glucose or lactose sugar.

ella species fermented glucose rapidly, the glucose prevented oxidation of ascorbic acid in every instance. On the other hand, since the *Salmonella* species do not attack lactose, this substance had no influence on the total decomposition of vitamin C by these species. The usual period for total oxidation of ascorbic acid was around 15–22 hours. When another substrate present with the vitamin was fermented within this time the vitamin usually was spared completely from oxidation. When a given carbohydrate spared the vitamin from oxidation in a

bacterial culture the protection offered was, as a rule, 100 per cent; whereas, if there was no protection the decomposition brought about by bacteria also was 100 per cent.

These studies demonstrate a rather familiar observation in bacterial metabolism. Some bacteria seem to have a preference for certain substrates when two or more fermentable substances are present in the same medium; one or more of the substances may be spared entirely from attack, while the others are readily utilized.

A study of the data in table 2 reveals another interesting observation. A comparison of the uninoculated controls for auto-oxidation of ascorbic acid with the cultures showing sparing of the vitamin by the various carbohydrates indicates a higher concentration of the vitamin remaining in the cultures than in the controls. It would appear that the increased protection in the presence of enormous numbers of bacteria was due to rapid removal of residual traces of oxygen in the medium by the organisms.

RELATION BETWEEN BACTERIAL GROWTH AND DECOMPOSITION OF VITAMIN C

Vitamin C as a carbon food source in synthetic medium

A series of experiments was designed to inquire into the ability of representative species of vitamin-C-decomposing bacteria to utilize ascorbic acid as sole carbon source in synthetic medium. A basal synthetic broth medium was prepared having the following composition:

NH ₄ Cl.....	5.0 gm.
(NH ₄) ₂ HPO ₄	0.5 gm.
Na ₂ SO ₄	5.0 gm.
MgSO ₄	0.1 gm.
KH ₂ PO ₄	1.0 gm.
K ₂ HPO ₄	1.0 gm.
Distilled water..	1,000.0 ml.

Ascorbic acid was added to the basal medium in concentrations of 10.00, 2.00, 0.8, 0.25, and 0 (control) mg. per ml. For comparative purposes growth was followed in glucose-basal-medium broth and peptone-basal-medium broth having the same concentrations of ascorbic acid as the synthetic medium. All media were buffered to pH 7.0, after adding ascorbic acid.

Four of the test organisms, *Escherichia coli*, *Aerobacter aerogenes*, *Proteus morgani* and *Salmonella aertrycke*, were able to grow in ascorbic-acid-basal broth in concentrations of 0.25, 0.8 and 2.0 mg. per ml., upon first transfer from nutrient broth. Growth was slow in synthetic ascorbic acid medium and, depending on the concentration of the vitamin, required anywhere from 3 to 7 days to develop sufficiently to destroy the vitamin completely. A vitamin concentration of 2 mg. per ml. was definitely inhibitory to all species studied, in spite of buffering of the medium at pH 7.0. Upon repeated transfer in ascorbic-acid-basal broth *Aerobacter aerogenes* was the only species which showed persistent ability to grow and decompose the substance. *Escherichia coli*, *Proteus morgani* and *Salmonella*

aertrycke failed to develop after the second transfer to ascorbic-acid-basal broth, indicating that growth and subsequent decomposition of the vitamin upon initial inoculation to synthetic broth probably was due to sufficient nutrient material that was carried over in the inoculum from the parent nutrient broth culture. The same four organisms were able to grow on continuous transfer in glucose-basal medium in which glucose was the only source of carbon; they also grew well when ascorbic acid was added to the glucose-basal medium in concentrations of 0.25, 0.8 and 2.0 mg. per ml., but growth was inhibited entirely when the vitamin concentration was increased to 10.00 mg. per ml. Glucose in the synthetic broth protected the vitamin completely from oxidation by the test organisms that were able to develop in the medium. *Streptococcus liquefaciens* apparently was unable to grow in the synthetic medium having NH_4Cl as the only nitrogen source, even when glucose was used as a carbon source; likewise, it failed to grow when ascorbic acid was used as the carbon source.

In spite of the buffering of the medium at pH 7.0, a concentration of 10 mg. ascorbic acid per ml. in basal broth or in glucose-basal broth inhibited growth completely in some instances, and actually killed off the inoculum in others; however, the addition of peptone to the media in 1 per cent amount appeared to remove the inhibitory and germicidal effects entirely and, as will be noted later, stimulated tremendous increase in growth over and above that observed in parallel controls which lacked the vitamin.

Influence of concentration of ascorbic acid in peptone broth on bacterial growth

The data in table 3 show a direct correlation between degree of utilization of ascorbic acid by bacteria and degree of growth. As indicated by galvanometer deflection values representing growth, increase in ascorbic acid concentration to the extent of 10.00 mg. per ml. broth brought about a tremendous increase in growth, compared with the vitamin-free controls. All five ascorbic-acid-fermenting species tested showed similar marked stimulation. Since concentration of ascorbic acid directly influences the degree of growth, and since the rate of growth correlates well with the rate of vitamin decomposition, being greatly accelerated when the cells are most viable, utilization of the vitamin as a carbon food source appears quite certain.

Three species of bacteria which are unable to utilize ascorbic acid, namely *Proteus* X-19, *Salmonella pullorum* and *Serratia marcescens*, were not affected significantly by differences in concentration of the substance in peptone broth.

MODE OF DECOMPOSITION

The course of ascorbic acid oxidation varies with the nature of the oxidizing agent. Normal oxidation of vitamin C in plant tissues, catalyzed by enzymes, has been shown by Szent-Gyorgyi (1931) to be a reversible oxidation to the dehydro state. On the other hand, according to Barron *et al.* (1936) and Kellie and Zilva (1935), atmospheric oxidation of the vitamin in an alkaline solution proceeds under the influence of cupric ions to a more complete irreversible stage. Decomposition of ascorbic acid by bacteria also is carried beyond the reversible

dehydro state. As shown in table 1, numerous cultures of various organisms which had removed the entire amount of ascorbic acid added to the medium in the reduced state failed to show any recovery of vitamin C in the reduced form upon treatment with hydrogen sulfide and subsequent removal of the sulfuretted gas with nitrogen. If the oxidation had been merely a reversible one, through the dehydro state, hydrogen sulfide would have reduced the oxidized product, restoring it to its original form.

TABLE 3

Influence of concentration of ascorbic acid in peptone broth buffered at pH 7.0 upon growth of ascorbic acid-decomposing and on ascorbic acid-non-decomposing bacteria

ASCORBIC ACID-DECOMPOSING SPECIES INCUBATED UNDER ANAEROBIC CONDITIONS AT 37°C.	CONCENTRATION OF ASCORBIC ACID								
	0.0 mg./ml.			0.8 mg./ml.			10.00 mg./ml.		
	0 hours	10 hours	36 hours	0 hours	10 hours	36 hours	0 hours	10 hours	36 hours
<i>Escherichia coli</i> (Dd)	5*	30	35	5 +†	44 —	57 —	5 +	50 +	58 —
<i>Aerobacter aerogenes</i> (5A5) .	6	20	37	5 +	33 —	44 —	5 +	47 +	82 —
<i>Proteus morgani</i> (Ba) . . .	3	15	40	4 +	31 —	50 —	4 +	33 +	74 —
<i>Salmonella aertrycke</i> . . .	2	19	23	2 +	34 —	44 —	2 +	57 +	76 —
<i>Streptococcus liquefa-</i> <i>ciens</i>	3	19	19	2 +	34 —	34 —	4 +	49 +	78 —
Non-decomposing species									
<i>Proteus</i> X-19	3	11	12	4 +	7 +	10 +	4 +	7 +	10 +
<i>Salmonella pullorum</i> (P-54)	4	15	16	3 +	12 +	16 +	7 +	10 +	10 +
<i>Serratia marcescens</i> (C-20)	2	13	14	4 +	12 +	14 +	4 +	12 +	14 +

* Units galvanometer deflection of Evelyn photoelectric colorimeter indicating turbidity and indirectly degree of growth.

† + indicates presence of ascorbic acid in medium. — indicates complete bacterial decomposition of the vitamin.

Gas production during the fermentation of carbohydrates usually indicates a rather complete decomposition process. Accordingly, all of the species showing ability to decompose ascorbic acid were inoculated into peptone ascorbic-acid broth contained in large Durham fermentation tubes, to determine the production of gas during the decomposition process. Concentrations of 4.0 and 2.0 mg. ascorbic acid per ml. broth were employed, and all cultures incubated in duplicate under anaerobic conditions at 37°C. As shown in table 1, organisms which

usually produce gas in carbohydrate medium (members of the colon-aerogenes and *Salmonella* groups) also generate gas during the fermentation of ascorbic acid; on the other hand, species of *Streptococcus* and *Vibrio*, as well as *Eberthella typhosa* and *Salmonella gallinarum*, which are normally non-aerogenic, likewise failed to produce gas during the oxidation.

Periodic quantitative tests for ascorbic acid in the duplicate culture series showed a positive correlation between increase in gas production and decrease in the vitamin content of the medium. The amount of gas production also directly corresponded with the amount of vitamin substrate added to the medium. Parallel control cultures lacking the vitamin substrate showed no gas production.

Dehydrogenase studies. Filtrates from heavy cultures of vitamin-C-decomposing bacteria showed no effect on ascorbic acid under either aerobic or anaerobic conditions, regardless of age of culture; thus it seems that the factor responsible is not an exoenzyme. The present studies have demonstrated that the decomposition occurs rapidly under anaerobic conditions in the presence of living, actively metabolizing cells, and that the activation of the substrate in the absence of molecular oxygen implies the operation of a dehydrogenase system.

The "initial capacity" of the organisms to attack ascorbic acid and the "all or none" type of the response suggest that the endoenzyme involved is constitutive rather than adaptive in character. A series of experiments was carried out in which both vitamin-C-decomposing and non-decomposing species of bacteria were exposed to ascorbic acid substrate in nutrient broth by frequent transfer for varying periods up to three and one-half months. It was found that daily transfer of vitamin-C-decomposing species over a period of only five days resulted in marked acceleration of the rate of decomposition, but this "adaptive" characteristic was only temporary. Non-decomposing species exposed to ascorbic acid substrate over a period of several weeks failed to respond adaptively.

The operation of a dehydrogenase system may be detected easily under anaerobic conditions by the use of an appropriate oxidation-reduction dye indicator, the substrate in question and washed "resting cells" of the bacterial species to be examined. Accordingly, an experiment was planned to detect transfer of hydrogen by bacteria from ascorbic acid substrate to a dye-indicator acting as hydrogen acceptor. The study of catalytic transfer of hydrogen by bacteria usually is carried out by the use of the well-known technique of Thunberg in which methylene blue is employed as artificial hydrogen acceptor. In selecting an oxidation-reduction indicator for the present study it was found that methylene blue could not be used, because it is reduced by the reduced form of ascorbic acid, the rate of reduction depending on the concentration of the reagents involved and the temperature at which the reaction takes place. It was observed also that methylene blue in the concentration required was toxic to some of the test organisms, especially the enterococci. Nile blue proved to be a satisfactory dye-indicator, since it is stable toward large concentrations of ascorbic acid, and in relatively low dilution is not toxic to bacteria. The value of oxidation-reduction dye-indicators like methylene blue and nile blue lies in the fact that these dyes exist in a reduced form which is colorless and in an oxidized form which is blue. Optimum concentrations of the reagents employed were determined by

preliminary experiments, and tests with appropriate controls were carried out under anaerobic conditions at 37°C. A positive test was indicated by complete reduction of nile blue to the colorless state within 60 minutes, providing the parallel control lacking vitamin substrate was not also reduced in that period of time.

The results of the nile blue reduction studies, presented in table 1, show that all of the species of bacteria which are able to decompose ascorbic acid in nutrient broth also activate the vitamin under "resting cell" conditions in the presence of nile blue, thus suggesting the action of an ascorbic-acid dehydrogenase. However, the following organisms which did not attack ascorbic acid in nutrient broth activated the substrate in the presence of nile blue acceptor and reduced the dye: various strains of *Salmonella pullorum*, *Proteus vulgaris*, *Serratia marcescens*, and species of *Bacillus*. Parallel tests in which glucose was used as substrate gave similar results with all organisms tested; however, the rate of reduction was usually a little more rapid than when ascorbic acid was employed as substrate. Most reductions occurred within 20-30 minutes.

From the present observations one might postulate that nile blue acts as a readily available hydrogen acceptor in the oxidation of vitamin C by organisms such as *Salmonella pullorum* and *Proteus vulgaris*, which are unable to attack the substance under ordinary conditions in nutrient medium. Perhaps these organisms do not provide the appropriate hydrogen acceptor or other essential component in the dehydrogenase system, under the conditions of growth in a nutrient medium. Failure of members of the *Alcaligenes* group to activate either glucose or ascorbic acid substrate is in keeping with their asaccharolytic nature and apparent deficiency in dehydrogenase enzyme systems.

Experiments were carried out to determine the effect of various enzyme inhibitors and growth-inhibiting substances on the activation of ascorbic acid in the resting-cell nile-blue system. Sodium fluoride, arsenious oxide, urethane and sodium selenite have been reported by different investigators as arresting the action of various types of dehydrogenases, especially sodium selenite, which seems to affect specifically the dehydrogenases. Sodium selenite (0.01M; 0.001M; 0.0001M) inhibited completely the action of ascorbic acid dehydrogenase of three test organisms, *Escherichia coli*, *Aerobacter aerogenes*, and *Proteus morgani*; viability tests showed that sodium selenite in the concentrations employed does not prevent the growth of these organisms. Potassium cyanide (0.01M; 0.001M), sodium fluoride (0.01M; 0.001M), arsenious oxide (0.02M; 0.002M) and urethane (0.03M; 0.02M) failed to inhibit dye-reduction significantly. These substances when added to nutrient broth in similar concentration did not prevent bacterial growth, but in some instances growth was retarded, depending on the concentration of the inhibitor. Vitamin C added to nutrient broth containing these substances in similar amount was completely destroyed in every case, after good growth was evident, indicating again no inhibition of ascorbic acid activation in nutrient broth. Acetone (5 per cent; 2.5 per cent), toluene (5 per cent; 2.5 per cent) and chloroform (5 per cent; 2.5 per cent) inhibited the reduction of nile blue by the three test species in the presence of ascorbic acid; viability tests showed these agents to be inhibitory to growth or germicidal in the concentrations indicated.

In the light of the present observations, it appears that sodium selenite specifically inhibits ascorbic acid dehydrogenase; even though the substance was not toxic to the cells, it prevented the action of the enzyme. The cells must be living, but not necessarily multiplying, to effect activation of ascorbic acid dehydrogenase in the Nile blue system; killing the cells with chemical agents prevented activation, whereas holding the cells at 46°C. to prevent cell growth greatly increased the rate of reduction. On the other hand, under the conditions common to bacterial decomposition of vitamin C in nutrient broth, the cells must be living and also actively multiplying. The following findings support this assumption: (1) holding heavy cell suspensions at 46°C. for several days, to prevent growth without extensive killing of the cells, showed no decomposition of the substance; (2) freezing and thawing massed cells several times, bringing about death and disruption of the majority of the cells, failed to accelerate the oxidation when the cells were resuspended in ascorbic acid nutrient broth; (3) when incubated at 37°C. an incubation period of at least 10–15 hours was required for complete destruction, regardless of the amount of inoculum added; and (4) any chemical factor which prevented or retarded growth of the organisms also prevented or retarded the oxidation of vitamin C.

DISCUSSION AND SUMMARY

The property of decomposing vitamin C is quite common among the enteric bacteria, including the intestinal streptococci.

In the presence of appreciable amounts of easily fermented carbohydrate, like glucose, the vitamin is protected from microbial decomposition, whereas in the absence of the competitive agents the ascorbic acid content of the medium becomes depleted rapidly.

Bacterial decomposition proceeds under anaerobic conditions. Bacteria which do not attack the vitamin exert a sparing action on this substance under ordinary aerobic conditions by removing atmospheric oxygen, and thus preventing auto-oxidation. Usually, such organisms grew better in the presence of the vitamin than in its absence, due presumably, at least in part, to the creation by this chemical agent of a lower and more favorable reduction potential.

Ascorbic acid was decomposed and utilized readily as a carbon food for the attacking bacteria, when the medium contained a suitable source of organic nitrogen, like peptone. This was indicated by the early disappearance of the vitamin and the large increase in bacterial growth over that observed in parallel vitamin-free controls. In the absence of a suitable nitrogen source, as for example in synthetic ammonium-chloride ascorbic-acid medium containing 10 mg. per ml. of the agent, no growth took place, and the bacterial cells were killed, in spite of buffering of the medium at pH 7.0. At lower concentrations of ascorbic acid (2 mg. or less per ml.) *Aerobacter aerogenes* alone was able to develop and decompose the substance.

Oxidation of the vitamin was in every instance carried beyond the reversible dehydro stage of decomposition. Furthermore, vitamin-C-decomposing bacteria which produced gas from ordinary carbohydrates also brought about gas formation in their action upon ascorbic acid.

Filtrates of active bacterial cultures did not possess the vitamin-C-decomposing property, the destruction being effected only by actively metabolizing microbial cells. These observations, together with the demonstration that the carrying to completion of the process in the absence of molecular oxygen, pointed to the operation of a dehydrogenase in the destruction of the vitamin. A dehydrogenase was demonstrated by the use of bacterial "resting cells" in a Nile-blue indicator system having ascorbic acid as the substrate.

Results of the present study lend further support to the assumption that under certain conditions vitamin C may be destroyed in the intestinal canal by bacteria, and thus lead to vitamin C deficiency. It would seem that this action cannot be of serious moment in the population at large, otherwise there would be more extensive clinical evidence of such deficiency. No doubt such factors as the small daily amount of ascorbic acid required by the normal human adult, the site and the rapidity of absorption from the intestine, enteric infection, diet, type of intestinal flora and dominance of vitamin-C-destroying bacteria, are definitely related to loss to the body by bacterial action. It may well be that bacterial destruction of ascorbic acid in the intestine becomes especially significant in certain pathological conditions where the vitamin, in addition to its essential rôle in normal metabolism, serves to play an effective part in recovery. Thus, in the healing of bone fractures or wounds in general, in certain conditions of shock, hypersensitivity, infection and poisoning due to various industrial chemicals the bodily demand for vitamin C is so increased that bacterial destruction of the substance and loss to the body by perspiration or by any other factor becomes exceedingly important (see Holmes, 1942).

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THE CHARACTERISTICS OF *PROTEUS AMMONIAE*

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There is considerable interest at present in the definition of the species comprising the genus *Proteus*. Particularly for the clinical bacteriologist, this genus has been a catch-all for strains which did not fit the genus *Salmonella* or the coliform group. The generic characters of *Proteus* are distinctive enough, but no agreement has been reached as to the valid species within the genus.

In the course of certain studies of gram-negative, non-lactose-fermenting bacteria we have examined 32 strains³ which we identified as *Proteus ammoniae* (Hager and Magath, 1925; Magath, 1928). Two of these strains were kindly furnished us from Magath's collection by Dr. L. Thompson, and may thus be regarded as authentic representatives of the species. The sources of the strains were as follows:

Cystoscopic urine samples.....	13
Stool specimens.....	9
Amer. Type Culture Coll.....	2
Magath's collection.....	2
Mislabeled stock cultures.....	2
Mastoidectomy.....	1
Guinea pig autopsy.....	1
Uncertain.....	2
Total.....	32

The largest single group came from the urinary tract, and it is probable that others would be listed in this group if complete records were available.

The reactions of the strains are shown in table 1. The strains agreed closely in their characteristics; all exceptions have been noted in the footnotes to table 1. Fermentations were determined in a medium of 1 per cent tryptose, 0.5 per cent carbohydrate, and brom-cresol-purple indicator, autoclaved for 10 minutes at 10 lbs. in 2 ml. volumes in 13 x 100 mm. tubes in hardware cloth racks. Fermentation of lactose and sucrose was also tested in media containing 5 per cent of the carbohydrate. Litmus milk was made up from fresh skimmed milk and was sterilized by the intermittent method. Indole was determined in 1 per cent tryptone water cultures by Kovacs' (1926) method. Acetyl-methyl-carbinol was determined in cultures grown in Bacto MRVP medium for 2 days at 37°C., using the reagents of Vaughn and Levine (1942). Citrate utilization

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³ We are pleased to acknowledge the assistance of Sol Haberman, Ph. D., Bacteriologist, Baylor Hospital, in the collection of these strains.

was determined on BBL Simmons Citrate Agar slants, and tartrate utilization in BBL Tartrate Agar stabs. Gelatinase action was determined with saturated ammonium sulfate on slants of Bacto Stone's Gelatin Agar after 2 days growth. Motility was observed in hanging drop and in Bacto Motility medium. Urease activity was demonstrated by the methods of Rustigian and Stuart (1941).

All strains grew readily on BBL Desoxycholate-Citrate plates and on Bacto MacConkey and S-S plates. The colonies on S-S plates had the distinctive black centers and sulfurous odor which often are indicative of the *Salmonella* group.⁴ Colonies on Bacto Bismuth Sulfite plates were small and green. Swarming was observed on plates of Tryptose agar with or without 5 per cent human blood, and also on plates of eosin-methylene-blue agar made by the formula of Stuart, Wheeler and Griffin (1938).

TABLE 1
Reactions of 32 strains of Proteus ammoniae

Adonitol.....	-	Maltose.....	-	Gas.....	+
Aesculin.....	-	Mannitol.....	-	Milk.....	alk.
Arabinose.....	-	Mannose.....	-	Casein.....	pept.
Cellobiose.....	-*	Alpha-methyl glucoside	-	Litmus.....	red
Dextrin.....	-	Raffinose.....	-	Indole.....	-
Dulcitol.....	-	Rhamnose.....	-	V.P.....	-
Galactose.....	+	Salicin.....	-	M.R.....	+
Glucose.....	+	Sol. starch.....	-	Gelatin ..	+
Glycerol	+	Sorbitol.....	-	Motility ..	+
Inositol.....	-	Sucrose $\frac{1}{2}$ %.....	-†	Sulfide.....	+
Inulin	-	Sucrose 5%.....	+	Citrate.....	+¶
Lactose $\frac{1}{2}$ %.....	-	Trehalose.....	+§	Tartrate.....	-
Lactose 5%.....	-	Xylose.....	+	Urea	+
Levulose	-†			Swarming	+

* Fermented slowly by 1 strain.

† Slow fermentation by 3 stool strains.

‡ Slow fermentation by 3 strains.

§ Two strains negative.

¶ One strain negative.

Preliminary observations indicate that this collection of strains is serologically heterogenous, in contrast to its slight bacteriological dissimilarities. Antigenic analysis according to the system established for the *Salmonella* group should be fruitful.

DISCUSSION

Proteus ammoniae possesses the generic characters of *Proteus*; namely, swarming, proteolysis, urea decomposition, fermentation of sucrose, and failure to ferment lactose. Sucrose fermentation is evident only after prolonged incubation, but appears readily if the concentration of sucrose in the medium is increased to 5 per cent. Hager and Magath (1926) state that the species belongs

⁴ This reaction has also been observed with certain "intermediate" coliform strains.

in the *Proteus* group despite the failure of their cultures to ferment sucrose. Our findings confirm their judgment by demonstrating delayed fermentation of this carbohydrate.

Our observations extend the description of this species. The positive citrate and sulfide reactions, and the failure to ferment adonitol, aesculin, cellobiose, mannose, alphanethylglucoside, raffinose, soluble starch and sorbitol, have all been unreported previously. The accepted description lists the species as indole-positive. All our cultures were indole-negative, including the 2 kindly furnished by Dr. Thompson.

While the serological heterogeneity of the species precludes its recognition by serological methods, it is bacteriologically recognizable as a *Proteus* except that it appears to be sucrose-negative because of the delayed fermentation of this sugar. As it is negative in levulose, maltose, mannitol, salicin, and the indole test, it presents itself in the clinical laboratory as a fairly distinct entity, but one for which a specific identification is rarely supplied. It has been pointed out elsewhere (Harrison, Fulton and O'Brien, 1943) that the presence of this species in the urinary tract is associated with clinical conditions which are worthy of careful study. The recognition of the causative organism by the laboratory is essential in a study of its clinical significance.

Levine (1942) has stated that *P. ammoniae* is a synonym of *Proteus mirabilis*. While there is general agreement as to the characteristics of the genus *Proteus*, there are contradictory views as to the characteristics of *P. mirabilis*. Topley and Wilson (1936) state: "Hausser's subdivision of the *Proteus* group on the basis of morphology, rate of liquefaction of gelatin, and indole production, has been found impracticable." In their treatment of the genus these authors convey the impression that they make no distinction between *Proteus vulgaris* and *P. mirabilis*. In view of this uncertainty, it may be argued that there is not yet sufficient evidence to justify the elimination of some of the species of *Proteus*. For this reason we would be reluctant to agree that *P. ammoniae* is a synonym of *P. mirabilis*.

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NUTRITIONAL STUDIES ON STREPTOCOCCUS LACTIS

I. AN UNIDENTIFIED GROWTH FACTOR FOUND IN YEAST EXTRACT

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The use of micro-organisms in assaying the vitamin content of food products has resulted in attempts to develop media of known composition. As the medium becomes more highly purified, growth may be retarded or may fail completely. From time to time, therefore, new substances essential or stimulating to growth have been recorded. Certain unknown "growth-promoting" factors still exist, while some have been successfully identified. As Snell and Mitchell (1941) demonstrated, adenine and thymine are essential for *Streptococcus lactis*. Stokstad (1941) discovered, for *Lactobacillus casei*, a growth factor which was a dinucleotide or a mixture of nucleotides and which could be partially replaced by a mixture of guanine and thymine. Strong, Feeny, and Earle (1941) recorded the stimulating action of asparagine on *Lactobacillus casei*. In wheat flour, Wegner, Kemmerer, and Fraps (1942) found a highly stable stimulating substance for *Lactobacillus casei*. According to Pollack and Lindner (1942), glutamine or glutamic acid is essential for the lactic-acid bacteria. Feeny and Strong (1942) identified as 1-asparagine, the stimulating material found in several substances, while Bauernfeind, Sortier, and Boruff (1942) recorded the stimulating action of lecithin on *Lactobacillus casei*. Pollack and Linder (1943) studied in peptone, a substance that stimulated the growth of *Lactobacillus casei*. Although they did not identify this substance, they gave several of its chemical characteristics.

MEDIUM

The composition of the test medium was as follows: glucose 10 gm., sodium citrate 6 gm., adenine 10 mg., guanine 10 mg., xanthine 10 mg., uracil 10 mg., dl-glumatic acid 0.5 gm., d-cysteine 0.1 gm., dl-leucine 0.1 gm., dl-isoleucine 0.1 gm., l (+) histidine 0.04 gm., d-arginine 0.08 gm., dl-valine 0.16 gm., dl-lysine 0.2 gm., thiamin hydrochloride 100 γ , pyridoxin hydrochloride 100 γ , calcium pantothenate 100 γ , riboflavin 200 γ , nicotinic acid 100 γ , biotin 1 γ (S.M.A. concentrate #1000), folic acid¹ 0.005, K₂HPO₄ 0.5 gm., KH₂PO₄ 0.5 gm., MgSO₄·7H₂O 0.2 gm., NaCl 0.01 gm., FeSO₄ 0.01 gm., MnSO₄ 0.01 gm., distilled water 1000 ml.

The hydrogen-ion concentration was adjusted to pH 6.6–6.8. All vitamins were sterilized separately and added aseptically to the medium. Tubes contained 10 ml. and were sterilized at 15 pounds for 20 minutes.

¹ The folic acid used in this study was obtained from Dr. H. K. Mitchell, University of Texas.

ASSAY METHOD

Two strains of *Streptococcus lactis* were used in these studies with identical results except that one strain grew faster than the other. The cultures were carried in yeast glucose peptone broth. Transfer to the test medium was made by a 0.01 ml. loop directly from a 24-hour culture. Where growth resulted, at least five loop transfers were made through the test medium. The dilution resulting from this transfer would remove any contaminating material from the original medium. This method avoids any possible injury to the organism from washing with saline or water.

Growth was measured by visual turbidity and pH development.

RESULTS

The basal medium failed to support the growth of the organism. Whatever growth did occur was slow and sparse and failed upon transfer. The addition of other amino acids did not produce increased growth. The following amino acids, besides those already in the medium, were tested: dl-methionine, l-tyrosine, l-tryptophane, dl-phenylalanine, dl-threonine, l-proline, dl-serine, B alanine, glycine, asparagine, l-aspartic acid, l-hydroxy proline, l-cystine, dl (+)-alanine, and norleucine.

Conceivably, the substance required for growth might be a known vitamin. To test this possibility the following vitamins were added to the basal medium: inositol, 2-methyl-1-4-naphthoquinone, choline, and p-amino benzoic acid. Various combinations of amino acids and vitamins, as well as a complete list of both, were added to the substrate.

The addition of a yeast extract was found to stimulate the organisms, resulting in very good growth and acid development. The unknown substance appeared to be organic in nature, since ashing the extract over a bunsen flame completely destroyed the activity. An attempt was made to obtain the material in a somewhat purer state. Twenty grams of Difco yeast extract were dissolved in 250 ml. of distilled water; and excess of lead acetate was added to the mixture; and the precipitate was removed by filtration. (AgNO_3 , HgCl_2 , CuSO_4 , and ZnCl_2 have failed to precipitate the material in question.) The metallic ion was removed by H_2S . The active material was found in the filtrate, while slight to negative activity was found in the precipitate. Fuller's earth and darco charcoal failed to adsorb the factor at pH 2.0, 3.0, 6.6, or 10.5. The addition of $\text{Ba}(\text{OH})_2$ or of oxalic acid did not precipitate or destroy the activity. The material, though readily soluble in water, was insoluble in CHCl_3 , $(\text{CH}_3)_2\text{CO}$, $\text{C}_2\text{H}_5\text{—O—C}_2\text{H}_5$, $\text{C}_2\text{H}_5\text{OH}$ and CH_3OH . Attempts to concentrate the active substance from the water by adding alcohol were a failure. The active material was readily dialyzable through a cellophane membrane. Vacuum distillation at higher temperatures failed. After the substance had been placed under vacuum, the temperature was slowly raised from 100°C to 210°C . A receiving flask was placed in the system and immersed in ice water. As subsequent tests proved, the activity was destroyed by the heat. No active substance was found in the distillate or residue. Evaporation and examination under the microscope indicated a

mixture of crystals. Since many salt impurities would naturally be present, one would expect to find these various crystal structures.

The addition of CuSO_4 to the substance, followed by evaporation and solubility tests, indicates that a copper complex, if formed, must be classed as water-soluble and methyl-alcohol-insoluble.

Several preparations of the stimulating substance were made, but results were invariably the same. In all tests the liquid was concentrated to a volume of 20 ml., and 0.1 cc. was added to each 10 ml. of the medium.

Attempts to replace the unknown substance with known materials have failed. The addition of yeast nucleic acid had no effect. The addition of d-arabinose and/or d-galacturonic acid did not stimulate growth.

SUMMARY

An unknown substance in yeast extract is essential for the growth of certain strains of *Streptococcus lactis*. It is apparently not a known vitamin and could not be replaced by a combination of 23 amino acids tested. It is not precipitable by Pb, Ag, Hg, Cu or Zn salts. Fuller's earth or darco activated charcoal fails to adsorb it at various pH values. It is not soluble in any common lipid solvent. Heating to 210°C. under a vacuum destroys the activity.

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AN IMPROVED TECHNIC FOR GROWING MICROORGANISMS UNDER ANAEROBIC CONDITIONS

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Many workers have experienced explosions or fires with anaerobe jars which employ gaseous hydrogen and palladinized asbestos as a catalyst. After experiencing three explosions in the course of one year in our laboratory, it was decided to reinvestigate this particular technic for anaerobiosis. As a result, a new and safer technic has been evolved.

One fact was obvious, immediately. Some of the technics recommend evacuating the anaerobe jar to about 50 mm. of mercury (corresponding to a reading of about 710 mm. on a direct-reading vacuum gauge at sea level), and refilling the jar with hydrogen gas. This is supplying a volume of hydrogen in the jar which is more than thirty times that needed for combining with the residual oxygen in the jar. For example, in the usual anaerobe jar which has a volume of about two liters, only one-fifth of the atmosphere is oxygen, so there are only 400 ml. of oxygen, at the very most, in the jar. When evacuated as described above, at least 93 per cent of the gases in the jar are removed. Thus 93 per cent of the oxygen is removed, leaving only about 28 ml. When atmospheric pressure is reestablished with hydrogen gas, approximately 1860 ml. of hydrogen gas are added to the anaerobe jar to combine with the 28 ml. of oxygen which remain. Since hydrogen combines with oxygen in the ratio of two volumes of hydrogen to one of oxygen, the excess hydrogen in the jar is dangerously great. If the lid of an anaerobe jar should accidentally slip out of position, as sometimes happens when jars are being removed from the incubator, or a worker accidentally removes the lid, not knowing that the jar contains hydrogen gas, the sudden presence of air, along with the palladinized asbestos catalyst and the large volume of hydrogen gas, results in a fire or an explosion. In the technic which recommends evacuating the anaerobe jar to a residual pressure of 50 mm. of mercury, filling with carbon dioxide, re-evacuating to 50 mm. of mercury and filling with hydrogen, the remaining oxygen would be only about 2 ml. and the excess of hydrogen even greater than in the above example.

The second undesirable feature of the existing technics is that the inert gas, nitrogen, which constitutes approximately four-fifths of the atmosphere within the jar is replaced by highly explosive hydrogen gas. If it is desirable to evacuate and refill the anaerobe jar a few times in order to reduce the amount of residual oxygen in the jar, it would be preferable to use an inert gas instead of one which is highly inflammable.

The presence of 5 to 10 per cent carbon dioxide is known to be beneficial for the growth of many microorganisms, especially for primary isolation from the animal body. If the increased carbon-dioxide tension is not required for growth, its

presence is not known to be detrimental in any case. A mixture of 90 per cent nitrogen and 10 per cent carbon dioxide¹ was selected as the inert gas for "washing out" the jars. By the proposed technic, the final concentration of carbon dioxide in the anaerobe jar is nearly 9 per cent.

THE TECHNIC

(1) Cultures to be grown under anaerobic conditions are placed in any suitable anaerobe jar, *i.e.* any jar with a lid which makes an air-tight seal and which is fitted with a stop-cock. Under normal conditions we use glass jars with interchange aluminum lids with brass stopcocks. Because of the scarcity of strategic materials we find that a Pyrex desiccator and cover with standard taper sleeve works satisfactorily (Corning Glass Co., Catalog No. 3118).

(2) Into the jar, along with the cultures, are placed a dish containing a small amount of palladinized asbestos, about 0.3 to 0.5 grams, and a tube of decolorized methylene-blue indicator solution. For an indicator of anaerobiosis we employ three solutions: (a) 6 per cent aqueous glucose, (b) 0.25 per cent aqueous NaOH, (c) 0.015 per cent aqueous methylene blue. Equal parts of the three solutions are placed in a Pyrex test tube and heated to boiling, whereupon the blue color disappears and the solution becomes colorless. The decolorized methylene-blue solution is cooled by holding the tube under running cold water, then placed in the anaerobe jar.

(3) The lid is placed on the jar, an air-tight seal being insured by the use of a suitable stop-cock grease, the stop-cock opened, and connected by means of rubber tubing to an assembly which is connected to either a motor driven pump² or a water aspirator, a vacuum gauge, a cylinder of gas composed of 90 per cent nitrogen and 10 per cent carbon dioxide, and a cylinder of hydrogen gas.

(4) The jar is evacuated to a pressure of 152 mm. of mercury (a reading of 608 mm. on a direct-reading vacuum gauge, thus removing approximately four-fifths of the air in the jar), the vacuum line shut off, and the mixture of 90 per cent nitrogen and 10 per cent carbon dioxide allowed to flow into the jar until atmospheric pressure is established.

(5) The jar is evacuated to a pressure of 152 mm. of mercury, for a second time, and the mixture of 90 per cent nitrogen and 10 per cent carbon dioxide allowed to flow into the jar until atmospheric pressure is attained.

(6) The jar is evacuated to a pressure of 152 mm. of mercury, a third time, and the mixture of 90 per cent nitrogen and 10 per cent carbon dioxide allowed to enter the jar until atmospheric pressure is reached.

(7) The jar is evacuated to a pressure of 152 mm. of mercury, a fourth time, and the mixture of 90 per cent nitrogen and 10 per cent carbon dioxide allowed to flow into the jar until atmospheric pressure is equalized.

(8) The jar is evacuated to a pressure of 152 mm. of mercury, hydrogen gas is allowed to enter the jar until the pressure is increased 50 mm. to a reading of

¹ We have obtained our supply from the Ohio Chemical & Mfg. Co.

² We have found the motor driven Gast portable rotary air blast and suction apparatus, #1033-G, Arthur H. Thomas Co., Philadelphia, very good for this purpose.

202 mm. of mercury, then the mixture of 90 per cent nitrogen and 10 per cent carbon dioxide is allowed to enter the jar until the pressure is 20 mm. below that of the atmosphere. The stop-cock on the jar is closed, the jar disconnected from the assembly and placed in the incubator. Only three or four minutes are required to place a jar under anaerobic conditions.

(9) To open the jar, evacuate to a pressure of 152 mm. of mercury, allow air to enter the jar until atmospheric pressure is established and then remove the lid. The palladinized asbestos should be heated over a Bunsen burner for a short time before being returned to a stoppered stock bottle.

COMMENTS

Evacuating the jar to a pressure of 152 mm. of mercury was employed because (1) there is no danger of fresh agar being pulled away from the Petri dishes; (2) this pressure is easy to attain with a water aspirator or a less expensive motor driven vacuum pump; (3) it does not cause boiling of the recently heated tube of methylene-blue indicator for anaerobiosis; (4) it is four-fifths of the gaseous content of the jar.

Repeated evacuation and refilling of the jar never reduced the oxygen to the point where the methylene-blue indicator tube remained colorless indefinitely. It is necessary to employ the palladinized asbestos catalyst and a small amount of hydrogen gas.

Since such a small volume of hydrogen gas is employed (only enough to increase the pressure by 50 mm. of mercury), in some cases it would be possible for most of the gas to remain in the connections between the cylinder of hydrogen and the anaerobe jar without an appreciable amount entering the jar. For that reason, on the last evacuation a pressure of about 152 mm. of mercury is produced, the hydrogen added, and then more of the mixture of 90 per cent nitrogen and 10 per cent carbon dioxide added to flush all the hydrogen out of the connections and into the jar. Connections between the cylinders of gases and the apparatus should be as short as possible.

A final pressure of nearly 20 mm. of mercury below normal atmospheric pressure is allowed to remain in the jar to allow for the expansion of the gases when the jar is placed in the incubator and the temperature of its contents is raised from room temperature to 37°C.

The excess hydrogen in the jar is not sufficient to cause an explosion or fire if it comes into contact with the air, even in the presence of the catalyst. To test this point of safety, the lids have been removed suddenly from the jars and nothing happened. As a routine measure in the laboratory, however, we make it a practice to evacuate the jars to a pressure of 152 mm. of mercury, then allow air to enter before we remove the lids. One does not have to worry about an explosion if the air is allowed to enter too rapidly, if the lid should slip off when the stop-cock grease is warm, or if someone accidentally removes the lid while the jar is still under anaerobic conditions.

There appears to be no significant difference in the appearance or amount of growth of anaerobic microorganisms, whether the jar be filled with hydrogen gas

or if the jar contains a large portion of nitrogen and an amount of hydrogen gas only slightly in excess of that needed for the removal of the last traces of oxygen.

SUMMARY

1. An improved technic has been described for growing microorganisms under strict anaerobic conditions.
2. In addition to providing an atmosphere devoid of oxygen, the technic also supplies carbon dioxide in a concentration of nearly 9 per cent.
3. The technic is safer than other technics employing hydrogen gas and palladinized asbestos as a catalyst and is thought to be as safe as it is possible to make such a technic.
4. It is efficient and fairly rapid to perform, requiring only three or four minutes to put a jar under anaerobic conditions.

MICROBIOLOGICAL ASPECTS OF PENICILLIN

II. TURBIDIMETRIC STUDIES ON PENICILLIN INHIBITION

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The character of bacterial growth-inhibition as a function of penicillin concentration when influenced by imposed conditions may have a bearing on the nature of penicillin action. Turbidimetry has proved of some value in this respect. Indeed, this method appears to be the only feasible manner of analyzing certain effects. A satisfactory method for the quantitative estimation of penicillin based on the proportional inhibition of bacterial growth measured turbidimetrically has already been described (Foster, 1942), and a discussion of certain of its features has appeared in the preceding paper of this series (Foster and Woodruff, 1943).

The main portion of this paper deals with various applications of turbidimetry to the study of *in vitro* penicillin effects. Supplementary data pertaining to the procedure itself and certain other findings are also presented.

EXPERIMENTAL

Influence of pH on penicillin inhibition

In the previous paper (Foster and Woodruff, 1943), penicillin activity on *Staphylococcus aureus* H showed a clear-cut difference according to the pH of the medium when tested by the plate method. It appeared to be more than three times as great at pH 5.5 as at 7.0. To obtain the relations more quantitatively, a similar pH experiment was set up in liquid media and the inhibition measured turbidimetrically. Unless otherwise specified all experiments were done with 10 ml. broth per tube. Series of nutrient broth tubes were adjusted with H₂SO₄ and NaOH to pH 5.5, 6.5, 7.0, and 7.5, respectively, supplemented with one per cent of the appropriate phosphate buffer and with various levels of penicillin, inoculated and read as usual after 17 hours. Figure 1 shows that the initial pH did not alter significantly the shape of the inhibition curves nor the total degree of inhibition finally obtained. The latter portions of the pH 6.5 and 7.0 curves tended to deviate from a true logarithmic inhibition. Decreasing pH tended to lower somewhat the position of each whole curve on the scale. Probably, color changes in the medium due to the pH account for this shift. In none of the treatments was there any appreciable change in pH as a result of growth.

From these growth curves it is possible to calculate the inhibitory power of penicillin as a function of pH, or, expressed differently, that amount of penicillin causing 50 per cent inhibition at different pH's. The values listed below from two experiments fail to corroborate the findings obtained by the plate method;

that is, except for experimental deviations the same amount of penicillin is required to produce 50 per cent inhibition irrespective of pH. Certainly there is no regular effect associated with pH.

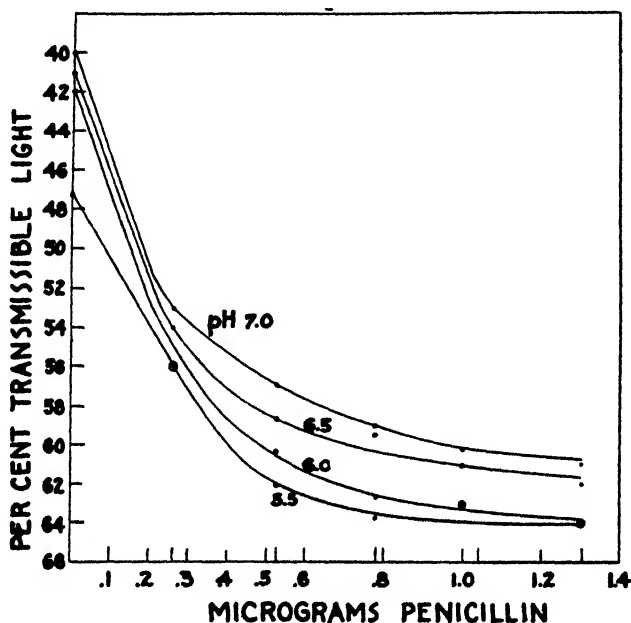


FIG. 1. pH AND PENICILLIN INHIBITION OF *S. AUREUS* H
The penicillin contained 15 Florey units per mg.

Penicillin required for 50 per cent inhibition

Experiment I

pH	Florey units/ml.
7.0	0.0031
6.5	0.0030
6.0	0.0031
5.5	0.0038

Experiment II

8.0	0.0048
7.5	0.0072
7.0	0.0060
6.5	0.0051
6.0	0.0051
5.5	0.0035
5.0	0.0053

The reason for the contradictory conclusions obtained by the plate and turbidimetric methods has not yet been explained satisfactorily. The latter method is considerably more accurate and presumably approaches more nearly ideal conditions for such tests because it represents a homogeneous physiological

system. A conspicuous difference between the two methods is that of degree of aerobiosis. The tube broth culture is apt to be considerably more anaerobic than plate cultures. Regular penicillin inhibition curves were set up in broth at pH 7.0 and 5.5 each in very shallow layer (10 ml. in 125 ml. Erlenmeyer flask) and in small tubes to provide a good depth. The tubes were put in an anaerobic jar (pyrogallol and NaOH; in another experiment N_2 containing 5 per cent CO_2 was used to displace the air). Good aerobic conditions prevailed in the shallow layer, and growth in these cases was considerably better than in the anaerobic set. Three experiments of this kind failed to reveal any regular significant effect of oxygen tension on the nature of the penicillin inhibition of *S. aureus* H at the different pH's. A similar experiment with *Lactobacillus casei* also showed no difference between aerobic and anaerobic cultivation. It is

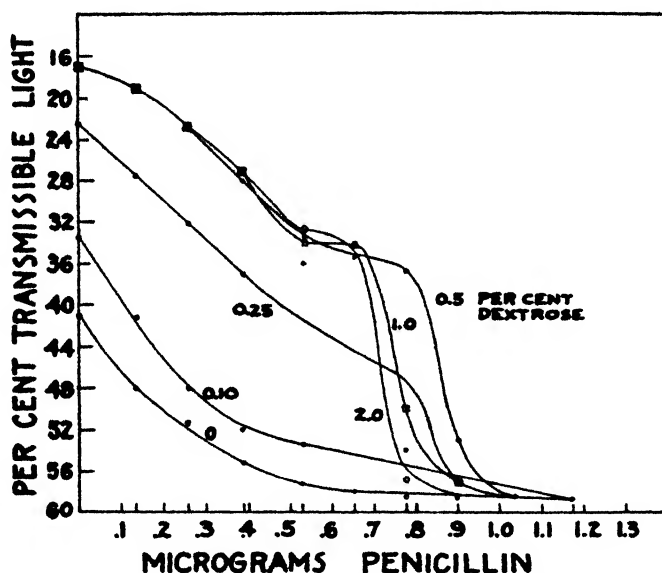


FIG. 2. GLUCOSE AND PENICILLIN INHIBITION
The penicillin contained 15 Florey units per mg.

possible that incidental factors such as a prolonged lag phase in the case of pH 5.5, diffusion effects, amount of inoculum, etc., could explain the pH effect obtained by the plate method as described in the preceding paper.

Influence of glucose

The addition of certain levels of glucose to nutrient broth leads to the appearance of peculiar irregularities in the inhibition curve (fig. 2). In all instances glucose was sterilized separately in aqueous solution for 15 minutes at $120^{\circ}C$. and added aseptically to the rest of the sterile medium. Glucose up to 0.25 per cent markedly increases the total amount of growth which *S. aureus* makes in a basal medium of regular nutrient broth buffered at pH 7.0 with 1 per cent phosphate (tubes with no penicillin, fig. 2). The control series with no glucose

and the 0.1 per cent glucose series show the usual concave curve of inhibition, and these are regular logarithmic inhibitions. The 0.5, 1.0, and 2.0 per cent series are typical glucose curves and show clearly that this sugar changes the character of the penicillin inhibition. The picture is practically identical in these three concentrations and the 0.25 per cent set is intermediate. Instead of the regular concave curve normally obtained, the rates of inhibition at different penicillin levels are altered so as to give a convex curve over the first half. Generally, at about the middle portion of the curves, the inhibition becomes irregular. There may be a peculiar break which makes a plateau indicative of less inhibition than should be expected, or more frequently the next small penicillin increments sharply evoke a very marked inhibition, this phase being at maximum

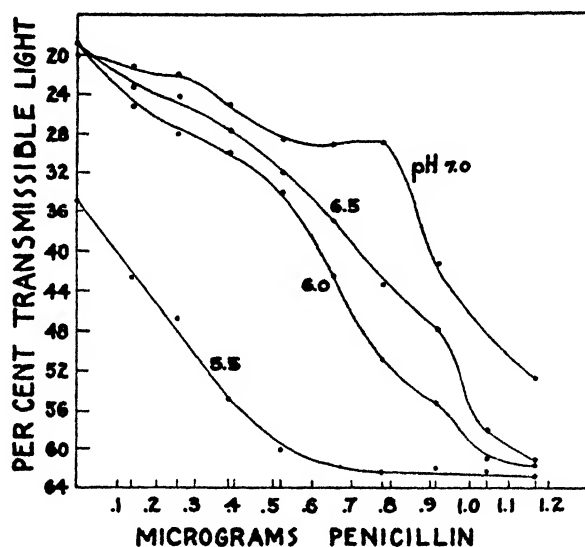


FIG. 3. INITIAL pH AND PENICILLIN INHIBITION IN GLUCOSE BROTH
The penicillin contained 15 Florey units per mg.

rate. The leveling off point of complete inhibition is considerably sharper than usual.

Irregular curves such as these have been obtained in glucose media numerous times, and although the course may not always be the same, the sharp mid-curve change in inhibition rate is invariably noted.

The interpretation of these events is not immediately obvious. The concavity of the first portion of the glucose curves, representing a relatively low rate of inhibition, possibly could be due to the fact that growth is limited by some variable in the medium, presumably a function of metabolism, or that penicillin is being inactivated. Since this portion represents most growth, such an effect would be most marked in this stage. Limitation due to acid accumulation and subsequent low pH's would appear possible. This point was the subject of a detailed experiment in which nutrient broth containing 1 per cent glucose was

buffered at pH 5.5, 6.0, 6.5, and 7.0, respectively, each with three different concentrations of phosphate buffer, namely, 1, 2 and 3 per cent. The usual penicillin inhibition curves were run and pH measurements made on each treatment. The 1 per cent phosphate curves are given in figure 3; 2 and 3 per cent phosphate curves lead to essentially the same conclusions.

At pH 5.5 maximum growth was about half that at the higher pH's and with the lowest buffer (1 per cent) the drop in pH to 4.7 just about became limiting for *S. aureus*. The acidity results from the fermentation of the carbohydrate which yields mainly acetic, succinic and lactic acids. In the pH 6.0, 6.5 and 7.0 series the irregular breaks described above were obtained. The plateaus do not appear at all in the 5.5 set and become more marked progressively to the 7.0 set.

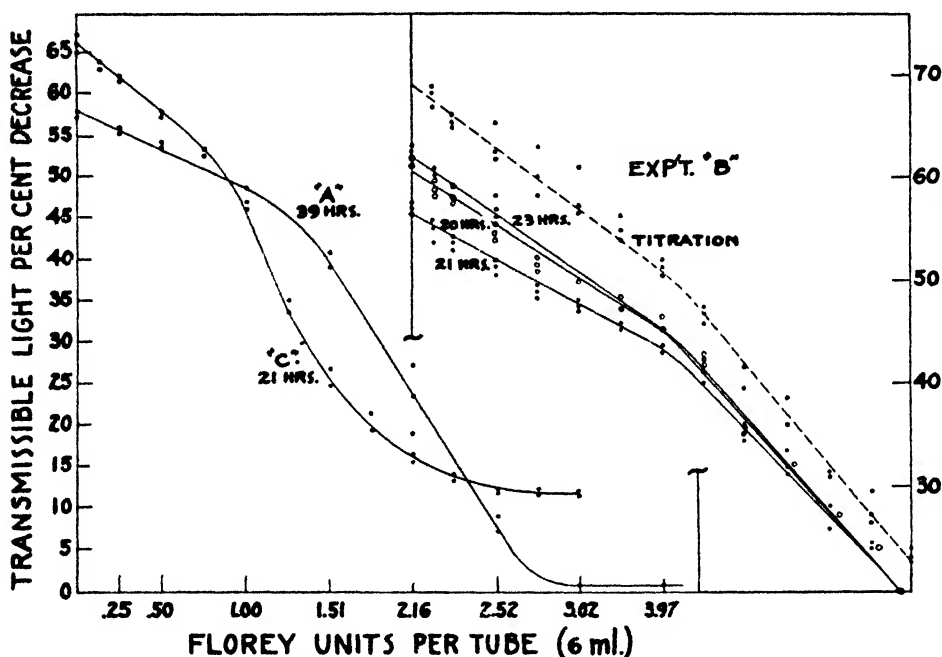


FIG. 4. PENICILLIN INHIBITION OF *LACTOBACILLUS CASEI*

Thus, pH apparently does have an effect on the penicillin inhibition curve, but so far this is detectable in broth cultures only in the presence of glucose. These plateaus are not directly associated with the occurrence of a specific limiting pH since in the different series they occur at different pH's which themselves are not limiting. Thus, it would seem that the plateaus are the cause and not the result of the pH changes.

Other bacteria also show the same general type of curve in media containing glucose. In some cases, however, the typical mid-curve break does not appear, so that the final picture shows a low steady rate of inhibition up to a certain point where it breaks more or less precipitously into a significantly increased rate of inhibition. Figure 4, curves A and C, shows the types of curves for

Lactobacillus casei obtained in two different experiments (39 and 21 hrs. incubation). In another experiment (B, fig. 4) the sharp break occurred at the same place after 16, 20 and 23 hours' incubation. The dotted line in figure 4 represents the curve of titratable acidity in 6 ml. medium in the 23-hour penicillin inhibition curve. The break in titration coincides with the break in turbidity read on those tubes before they were titrated. Since lactic acid formation is a function of growth, this is not unexpected. Other fermentable sugars, including maltose, sucrose, and lactose, gave breaks in the curves of *L. casei* similar to those of glucose.

Inhibition curves in glucose media after 20 hours' incubation at 37°C. are shown for *Streptococcus bovis* and *Streptococcus fecalis* in figure 5. The slow inhibition rate persisted over twice the range with *S. fecalis* as compared with *S. bovis*. With another crude penicillin preparation only $\frac{1}{6}$ the purity of that used above, the lag in inhibition was considerably shorter for both these same organisms. However, any one penicillin preparation gives different curves from day to day.

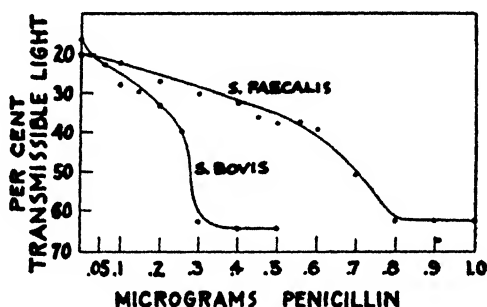


FIG. 5. PENICILLIN INHIBITION OF *S. BOVIS* AND *S. FAECALIS*

A number of other homofermentative lactic acid bacteria were found to be sensitive to penicillin in a rough quantitative survey. Those tested were *Lactobacillus casei* (2 strains), *L. delbrückii*, *L. arabinosus*, *L. bulgaricus*, *L. mesenteroides*, *Streptococcus lactis* (2 strains), *S. fecalis*, *S. zymogenes*, and *S. salivarius*. Of these, *S. salivarius*, *S. fecalis* and *S. bovis* were all in the same range of sensitivity as *S. aureus* H while the others were much less sensitive.

The presence of glucose favoring these abnormal inhibition curves suggests that the glucose-containing medium may in some manner interfere with the normal inhibition mechanism of penicillin or may modify or inactivate penicillin. The latter action would, of course, be occurring during the 16-hour incubation period, and the final inhibition curve would represent the resultant of growth occurring in a changing concentration of penicillin. Since pH has already been shown to influence the glucose effect, the final curve is also the resultant of changing pH.

Any modification or inactivation of penicillin in such a medium might be magnified with increased time. Three out of six series of tubes containing nu-

trient broth plus 1 per cent phosphate buffer at pH 7.0 received glucose (sterilized separately) to a final concentration of 1 per cent. Various levels of a Seitz filtered penicillin solution were added aseptically to the different tubes of two glucose and two nutrient broth sets so that each represented graded series of penicillin concentrations. One penicillin-containing set with and without glucose was allowed to preincubate uninoculated at 37°C., and a similar set at 2°C. The third pair of series without added penicillin was also stored at 2°C. After 48 hours, penicillin was added to the third pair and all six series were inoculated with *S. aureus*, incubated 18 hours further at 37°C. and the inhibition curves obtained. The results are summarized in figure 6. In the absence of glucose, 48 hours' preincubation at either 2° or 37°C. had no significant effect on penicillin inhibition as compared to the controls with preincubation. In the presence of

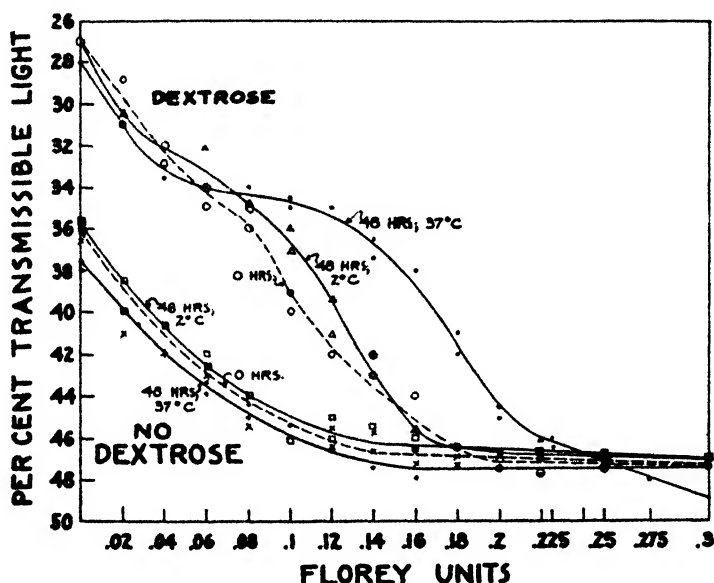


FIG. 6. PREINCUBATION AND PENICILLIN INHIBITION IN GLUCOSE BROTH

glucose, preincubation of the sterile solutions caused a clear-cut reduction in penicillin inhibition. The reduction is considerably more marked at 37°C. than at 2°C.; the latter inhibition curve is only slightly different from the controls without preincubation. The break in the zero time curve arises during the growth incubation period. As noted earlier, the breaks are confined to the middle portion of the curves. After preincubation as much as 60 per cent higher initial penicillin concentration was required to obtain the same degree of inhibition as in the non-preincubation controls.

The antibacterial power of penicillin is, therefore, appreciably reduced by the presence of glucose in nutrient broth in the absence of cells. Whether this represents destruction of a portion of the penicillin or an alteration in the penicillin molecule so that the resulting product is a less potent antibacterial sub-

stance remains to be established. The change apparently is a chemical reaction, which proceeds, therefore, more rapidly at 37°C. than at 2°C. It is also apparently influenced by pH (fig. 4) and is caused by other sugars.

Of special interest is the fact that the inactivation occurs only over a certain range of concentrations of penicillin (see fig. 6). Attempts to secure reduction in the potency of solutions of penicillin by incubation at 37°C. in the presence and absence of glucose have failed to yield any appreciable inactivation. Solutions containing final concentrations of 4 and 40 Florey units per ml. in 1 per cent glucose with and without nutrient broth after several days did not decompose any faster than controls. The solutions were assayed periodically by the Oxford cup method (Abraham, *et al.*, 1941). The effect may appear only under certain concentration conditions.

Resistance vs. inhibition theory

Two possible actions could account for the inhibition of *S. aureus* by penicillin. The bacterial inoculum may consist of cells of various sensitivities; that is, covering a spectrum of sensitivities. Thus, graded doses of penicillin will inhibit cells of correspondingly greater resistance, and those cells which grow in concentrations of penicillin just below that required for complete inhibition will, according to this idea, be considerably more resistant than those cells inhibited by lower concentrations. The alternative theory is that all the cells are essentially of the same resistance and that the action of graded doses of penicillin is to prolong logarithmically the generation time of every cell.

It follows from the first of these ideas that cells removed from the different levels of penicillin used in a standard curve after 16 hours' incubation will contain cells of different sensitivities. A standard curve series of cultures were set up as usual and after 16 hours the turbidity curve shown in figure 7 (curve I) was obtained. From this curve, portions of tubes A, B, C, D, and E were diluted aseptically with sterile nutrient broth so that the number of cells, as indicated by turbidity measurements, was equal to those in tube F undiluted. One-tenth of a ml. of each of these diluted cultures (including F) was used to inoculate identical standard penicillin series of nutrient broth tubes. After 16 hours' incubation at 37°C., the inhibition curves were all identical (fig. 7, curve II). If the different inocula contained cells of different resistances, then inoculum from tube F should show little if any inhibition in the secondary standard curve, and E, D, C, B, and A, respectively, should show progressively increasing inhibition, with that from A corresponding to the curve finally obtained. Actually, all are identical with A, which makes it likely that the cells used as inoculum obtained from different concentrations of penicillin were largely of the same resistance, thus ruling out the idea of selective inhibition and supporting the theory of prolongation of generation time.

Applications of turbidimetric method

An outstanding feature of the turbidimetric method is that its sensitivity permits measurement of relatively small differences in penicillin activities with

a reasonable accuracy. The amount of inoculum is without effect over at least a 1000-fold range. The following are some applications.

Decomposition of penicillin by pasteurization

Frequently, penicillin samples, especially culture filtrates from the mold, are contaminated with bacteria so that a determination of the penicillin content by any bacterial method is very much open to question. Not only do contaminating bacteria obscure the reading of the endpoint in the assay, but they are quite apt to contain or excrete the enzyme penicillase, which decomposes penicillin (Abraham and Chain, 1940). Hence, it is imperative to have a way of eliminating bacterial contaminants. Seitz filtration may be useful, but the sample may be too small or too large to filter effectively, and, besides, some losses through

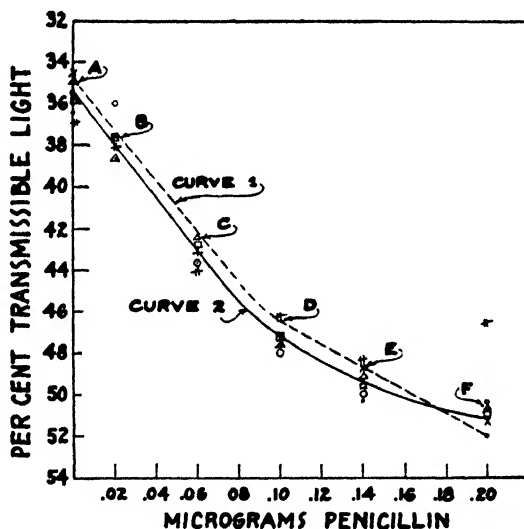


FIG. 7. PENICILLIN INHIBITION OF CELLS REMOVED AT DIFFERENT STAGES OF INHIBITION

adsorption may occur. In addition, the action of the soluble penicillase would continue.

Pasteurization is frequently the only feasible procedure, and it is, therefore, important to know whether penicillin deterioration occurs and how much. Penicillin is quite unstable at boiling temperature. Samples were diluted with sterile M/50 phosphate buffer at pH 7.2 to contain approximately 0.02 Florey units per ml. and pasteurized at 60°C. for 30 minutes. The solutions were then cooled immediately and assayed turbidimetrically (Foster, 1942). Unpasteurized controls were assayed simultaneously. The standard curve was run with pasteurized and unpasteurized standards. The data in table 1 show that pasteurization temperatures caused significant losses of activity in all preparations tested. The loss, when read against the regular (unpasteurized) standard, varies between samples, and this agrees with the general observation that different preparations of penicillin seldom behave exactly alike chemically or

physically. However, by reading pasteurized samples against the standard penicillin which has been similarly pasteurized, the losses are reduced and the values obtained are reasonably valid. In general, penicillin assays may be done under pasteurization conditions with a useful degree of accuracy provided the standard is also pasteurized.

pH stability of penicillin

Here again different samples may behave differently. However, the following experiment shows the characteristics of pH stability of penicillin. Twenty mg.

TABLE 1
Penicillin losses by pasteurization

SAMPLE	STANDARD UNPASTEURIZED		STANDARD PASTEURIZED	LOSS BY PASTEURIZATION	
	Unpast.	Past.	Past.	Calc. from unpast. stand.	Calc. from past. stand.
	<i>F.u./ml.</i>	<i>F.u./ml.</i>	<i>F.u./ml.</i>	<i>per cent</i>	<i>per cent</i>
11	0.020	0.016	0.016	20	20
12	.019	.016	.019	16	0
13	.030	.020	.024	33	20
14	.019	.016	.019	16	0
15	.019	.012	.016	37	16
16	.022	.018	.022	18	0
PENICILLIN BROTH					
A	9.7	9.1		6	0
B	2.0	1.7		15	
C	0.86	0.86		0	
SOLIDS					
	<i>F.u./mg.</i>	<i>F.u./mg.</i>			
1	74	69		7	
2	15	14.0		7	

of a sample containing about 10 Florey units per mg. was dissolved in 120 ml. of H₂O and 10 ml. of this solution added to 90 ml. portions of solutions adjusted and buffered (where possible) with the appropriate mixtures of H₃PO₄, KH₂PO₄, K₂HPO₄, Na₂PO₄ and NaOH to different pH's. The final pH's were 2.0, 2.6, 2.9, 4.8, 5.8, 6.8, 7.9, 9.4, and 10.3, respectively. Excess ether was added as a preservative and the solutions held at 28 to 30°C., samples being removed at intervals for turbidimetric assay. The samples were diluted immediately with ice cold phosphate buffer so that the final pH was 7.0 and then frozen in dry ice until assayed, which usually was done the same day. The results are presented graphically in figure 8. Penicillin is exceedingly labile at pH's below 4.8 and above 7.9, losing all activity in a matter of a few hours. Maximum stability in this experiment was at pH 5.8.

Short-time assay

In a previous paper, a short time (4 to 5 hour) assay based on the growth of *S. aureus* H in nutrient broth was described (Foster and Woodruff, 1943). A decided improvement in that procedure has since been made. Essentially, the

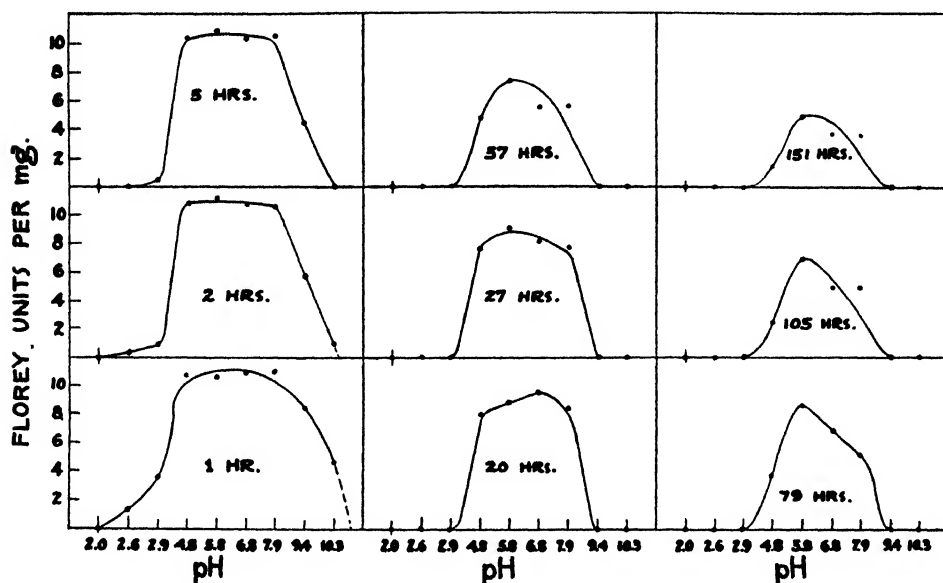


FIG. 8. pH STABILITY WITH TIME
Temp. = 28-30°C.

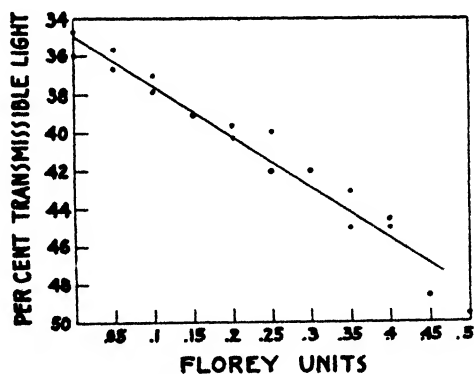


FIG. 9. STANDARD INHIBITION CURVE OF *B. ADHAERANS* AFTER SHAKING 4.5 HOURS

problem resolves down to securing the maximum growth obtainable per unit time. Cultures aerated by agitation on a shaking machine develop much more rapidly than corresponding stationary tube cultures. Glucose accelerates growth even more, but for reasons discussed earlier, its use is not recommended.

From many cultures tested, *Bacillus adhaerans* was selected as the test organism in the short-time assay, because it made most rapid growth under shake conditions and is just as sensitive to penicillin as *S. aureus* H. Figure 9 shows a penicillin inhibition curve after 5 hours under the conditions described above. Growth is inversely proportional to the amount of penicillin over at least an 8 to 10-fold range.

In practice the assay is run as follows. *Standard curve*: Five points in duplicate (0, 0.1, 0.2, 0.3 and 0.4 Florey units/10 ml.) define the curve although it is probable that three would suffice. Two hundred ml. of sterile nutrient broth are inoculated with 2.0 ml. of an overnight aerated broth culture of *B. adhaerans* (or *S. aureus*) and then distributed in 10 ml. amounts into 50 ml. Erlenmeyer flasks. The curve tends to flatten out near the end if the inoculum contains too few or slow growing cells. The standard penicillin solution containing 1.0 Florey units per ml. is stored in the refrigerator or, preferably, in dry ice. Quantities of 0, 0.1, 0.2, 0.3 and 0.4 ml. of the standard solution are added to the respective flasks in duplicate. *Samples*: Unknown samples are diluted to contain 1.0 Florey unit per ml. on the basis of the expected potency and 0.1, 0.2 and 0.3 ml. added in duplicate to flasks as above. All dilutions are made in advance and the penicillin added to the medium all at one time. Sterile glassware is not required; the flasks do not need to be plugged. The flasks are placed on a shaking machine at 37°C. for 3 to 5 hours, after which growth is stopped immediately by cooling in ice water or by adding a drop of disinfectant solution (5 per cent phenol or formalin). Turbidities are read in the Evelyn photoelectric colorimeter. The standard curve is obtained by plotting per cent transmissible light against penicillin concentration (fig. 9). Potencies of the unknowns are computed from the standard curve, the values obtained for the three levels being averaged. Values of samples assayed by the regular Oxford cup method and by the short-time turbidimetric assay are compared in the following tabulation:

SAMPLE	FLOREY UNITS PER ML.		PER CENT DIFFERENCE
	Cup	5 hour turbidimetric	
A (broth)	8.4	6.4	-23.7
B (broth)	13.2	8.5	-35.6
C (broth)	13.8	13.0	-5.8
D (broth)	15.6	16.5	+5.8
E (broth)	15.6	15.9	-1.9
F (broth)	19.8	19.5	-1.5
G (solid)	77 F.u./mg.	47 F.u./mg.	-38.9
H (solid)	132 F.u./mg.	102 F.u./mg.	-22.7
I (solid)	143 F.u./mg.	118 F.u./mg.	-17.5

While not furnishing as high an order of accuracy as desirable, this method has found valuable application in the production of penicillin.

SUMMARY

Contrary to results with the plate method, by turbidimetric measurements the same amount of penicillin is required to effect a 50 per cent growth inhibition of *Staphylococcus aureus* H in nutrient broth at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. The presence of glucose and other sugars in the medium causes irregular breaks in the inhibition curves. This is apparently due to an alteration or partial inactivation of penicillin which occurs when sterile penicillin and glucose broth solutions are allowed to stand before inoculation. The effect is not obtained in the absence of glucose. It also takes place during the regular 16-hour incubation period. The inactivation is less at 2°C. than at 37°C. and could be found only at certain concentration levels of penicillin. Penicillin inhibition of *S. aureus* H is due to logarithmic prolongation of generation time and not to selection of resistant cells. Small losses occur during pasteurization of penicillin solutions. The stability of penicillin is best between 4.8 and 7.9. It is inactivated very rapidly beyond these extremes. A short-time (3 to 5 hour) turbidimetric assay employing *Bacillus adhaerans* as test organism and accelerating growth by forced aeration (shaking) is described.

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NOTES

A NOTE ON NON-GROUP-A STREPTOCOCCI ASSOCIATED WITH HUMAN INFECTION

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Evidence that serious disease may result from infection with streptococci other than those of Lancefield Group A is accumulating. Lancefield (1940-41) has

TABLE 1

Non-group-A streptococci isolated from 23 cases of streptococcal infection

CLINICAL DIAGNOSIS	NO. CASES	AGE	SOURCE OF CULTURE	SEROLOGICAL GROUP	TERMINATION
dermatomyositis	1	adult	heart blood, muscle	B	fatal
post-operative meningitis	1	adult	spinal fluid	B	fatal
otitis media, secondary meningitis	1	child	ear, spinal fluid	B	fatal
otitis media, secondary meningitis	1	child	ear, spinal fluid	B	fatal
meningitis ¹	2	child	spinal fluid	B	recovered
emphysema	1	child	chest fluid	B	recovered
acute peritonitis	1	child	peritoneal fluid	D (Type Lanc-1)	fatal
acute pharyngitis ²	1	adult	throat	D (Type Lanc-1)	recovered
streptococcal septicemia	1	adult	adrenals, sinuses, abscess	D (Type Lanc-1)	fatal
meningitis	1	child	spinal fluid	D (Type Lanc-1)	fatal
sub-acute bacterial endocarditis ³	3	adults	blood culture	D (Type Lanc-3)	fatal
sub-acute bacterial endocarditis ³	1	child	blood culture	D (Type Lanc-3)	?
acute tonsillitis ⁴	1	adult	throat	D (Type H69D-5)	recovered
brain abscess ⁵	1	child	surgical drainage	F	fatal
ventricular block	1	child	spinal fluid	H	recovered
sinusitis ⁶	2	adults	throat, sinus	K	recovered
sinusitis ⁶	2	child	throat, sinus	K	recovered
brain abscess, meningitis	1	child	surgical drainage, spinal fluid	K	recovered

¹ Also isolated from blood culture of 1 case.

² Present in pure culture at onset, isolated 9 times over period of 7 weeks, also isolated from stool.

³ Total of 5 cases examined, 1 strain unclassified.

⁴ Few *Staphylococcus albus* only other micro-organism isolated.

⁵ Rough *Hemophilus influenzae* also isolated.

⁶ Predominant micro-organism in 3 cases, all in same family, present in pure culture in 4th case. Etiological significance not clearly established.

reviewed the earlier reports of such infections. More recently, Rantz (1942) Rantz and Kirby (1942) have reported a number of cases attributed to streptococci belonging to serological groups B through K. The cases noted in Table 1 were encountered over a period of two years during the course of a study involving the classification of several thousand strains of hemolytic streptococci.

The strains belonging to groups B and D merit brief attention. The group B

strains gave beta reactions and "double zone" phenomenon on 5.0 per cent horse blood agar. The post-operative meningitis did not respond to sulfanilimide therapy despite maintenance of satisfactory blood levels. The group D strains were classified by precipitation and agglutination of both the protein-like group and the carbohydrate type-specific components. This method will be described in detail elsewhere. Alpha, beta and gamma strains are frequently encountered in this group. The strain isolated from the fatal peritonitis was heat-resistant. Biological characteristics could not be correlated with serological type. The strains isolated from sub-acute bacterial endocarditis gave alpha reactions on 5.0 per cent horse blood agar. In one case, the micro-organism was recovered from the blood cultures over a period of four weeks. Three of the cases proved fatal.

These non-group-A strains represent but a small percentage of the total number of hemolytic streptococci isolated from human infection during these studies. They are of interest in that many of them were associated with fatal infections.

The diversity of clinical diagnoses encountered in this small series is in accord with the observation that non-group-A streptococci are frequently associated with non-respiratory streptococcal infections (Rantz and Kirby (1942)).

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DIGESTION OF CASEIN BY STAPHYLOCOCCI ON MILK AGAR CONTAINING SERUM

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Proteolytic zones surrounding the colonies of staphylococci are occasionally seen on the milk agar medium used in this laboratory to determine chromogenesis. These zones are for the most part narrow and indistinct and require more than 24 hours to appear. A marked increase in casein digestion was observed when the medium was enriched with serum. Tests were run with 175 strains of staphylococci of human origin to compare caseinolysis on serum milk agar with the coagulase and fibrinolytic activity of these organisms. The cultures were isolated from various pathologic specimens and from the nose, throat, scurf and urine.

Serum milk agar was prepared by adding 30 ml. of skim milk to 20 ml. of distilled water containing 1.5 g. of agar. The milk and the agar solution were sterilized separately at 121°C. for 10 minutes and were mixed with 50 ml. of serum

after cooling to 50°C. Pooled human serum obtained from serology specimens and sterilized by Seitz filtration was employed. Inoculations were made on the surface of the medium which was dispensed in petri dishes and the cultures were incubated at 37°.

Coagulase production was determined with citrated rabbit plasma which was added in 0.5 ml. amounts to 0.2 ml. of tryptose broth containing a suspension of the organisms to be tested. Overnight growths of cultures grown on a solid medium were used for this purpose and suspensions were made to approximate the opacity of an 18-hour broth culture of staphylococci. The tests were incubated at 37° and examined for coagulation at hourly intervals for 3 or 4 hours and again after overnight incubation.

Tests for fibrinolysis were run by the heat-precipitated fibrinogen method of Christie and Wilson 1941. Citrated rabbit plasma and the medium proposed by Chapman 1942 were used. The medium was allowed to harden in petri dishes and was inoculated in the same manner employed for serum milk agar.

Zones of clearing surrounding the growth of caseinolytic strains appeared on the serum milk medium usually after 24 hours of incubation but occasional

TABLE 1

Comparison of coagulase, fibrinolysis and caseinolysis tests with 175 strains of staphylococci

COAGULASE	FIBRINOLYSIS		CASEINOLYSIS	
	Positive	Negative	Positive	Negative
Positive (102)	91	11	82	20
Negative (73)	1	72	2	71
	Positive (92)		81	11
	Negative (83)		3	80

cultures required 48 hours to become positive. The zones of fibrinolysis on heated plasma medium were for the most part larger and were almost always present after overnight incubation. Plaque-like areas of clearing which protruded from the periphery of the zone of fibrinolysis and which were sometimes entirely separate from this zone were frequently seen.

The results of comparative tests presented in table 1 show that the ability of staphylococci to produce fibrinolysis and caseinolysis is similar to their coagulase activity. Since the coagulase reaction is generally believed to be the most reliable test for pathogenicity among staphylococci these results are of interest. Whether or not the disagreement in reactions shown by some culture strains may be significant in determining some factor of pathogenicity awaits further study. The mechanism of casein digestion which occurs in a medium rich in serum was not determined.

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MOTILITY IN THE GENUS ACETOBACTER

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The importance of the type of flagellation, polar or peritrichous, in separating the various groups of gram-negative bacteria frequently has been subordinated to other characteristics which, albeit essential in diagnosis of the bacteria comprising a given genus, do not contribute to a stable group taxonomy. While preparing a revision of the genus *Acetobacter* for the Manual of Determinative Bacteriology it became apparent that the type of flagellation of the motile species of *Acetobacter* is not widely known. Since further detailed study of the genus as a whole has been eliminated for the duration of the present emergency, it has been suggested that the author's information concerning motility of species of *Acetobacter* should be recorded to amplify observations and opinions reported in the literature and to establish the type of flagellation common to the motile species.

Zeidler (1896) was the first to record polar flagellation by a representative of the genus *Acetobacter*. Orla-Jensen (1909) created the genus *Acetimonas* for the acetic acid bacteria primarily on the basis of polar flagellation. Others who have stressed the type of flagellation in classification of these bacteria include Kluyver and van Niel (1936), Rahn (1937) and Vaughn (1942).

Observations conducted with 346 cultures of acetic acid bacteria isolated from souring fruits and fruit juices, wine, beer and vinegar stock, as well as vinegar, clearly indicate that all motile cultures of *Acetobacter* have polar flagellation. The motile cultures, stained by the method of Conn and Wolfe (1938), were found to have a single polar flagellum. Illustrations have been published by Zeidler (1898) and Vaughn (1942).

Motile species studied and recognized by the author include *Acetobacter aceti*, *A. pasteurianum*, *A. rancens*, *A. melanogenum* and *A. oxydans*. Although stained preparations of these species showed only one polar flagellum, the number obviously should be verified with the electron microscope.

Motility of the acetic acid bacteria studied, as with many other gram-negative bacteria, was so markedly influenced by the nature of the medium, temperature of incubation, incubation period and other factors that the author concurs with the opinion of Beijerinck (1911) who thought that if proper conditions were found, all acetic acid bacteria would be observed to be motile.

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VEGETABLE BACTERIOLOGICAL MEDIA AS SUBSTITUTES FOR MEAT INFUSION MEDIA

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Many workers have suggested vegetable media for the cultivation of various organisms, although none of these has been found entirely satisfactory as a general-purpose medium. From vegetable material alone we have been able to prepare media which we find equal to, or more satisfactory than, meat-infusion peptone broth or agar as general culture media. The vegetable materials have been cotton-seed meal, peanut meal, soy-bean meal, various whole and sprouted grains, beans and seeds.

A satisfactory method of preparation is as follows:

Materials

650 gms. vegetable meal
30 gms. papain
5 gms. Na_2S
4,000 ml. water

Dissolve the papain in about 500 ml. of water. Dissolve the sodium sulfide in 100 ml. of water, add it to the papain solution and allow to set for fifteen minutes. Then add the papain solution to the mixture of vegetable meal and water. The mixture is adjusted to pH 5 with hydrochloric acid and incubated overnight at 37°C. Clarify by filtration. The filtrate is adjusted to pH 7.6 and heated to boiling and filtered. The medium may then be distributed into flasks or bottles, autoclaved, stored and diluted as used. The yield of concentrated medium is about 3,500 ml. and contains 7-10% of total solids. For use, the concentrated medium may be diluted with water to about 14,000 ml. and 0.5% of NaCl added, resulting in a finished broth containing 2-3% solids.

The cost is only a fraction of that of meat-infusion broth and no added peptone is required. Autodigests have been made by omitting the enzyme and following the technique previously described. For some specific purposes, digests of

sprouted seeds and beans were found superior to digests of ungerminated plant materials.

Media made from different vegetable materials vary in their growth-promoting properties. Perhaps the most satisfactory single vegetable source is soy-bean meal. However, we recommend a mixture of soy bean and cotton seed or peanut meals for the preparation of general purpose media.

Vegetable media will grow many streptococci, pneumococci, gonococci and other fastidious organisms without the addition of serum or other growth-promoting factors. We have not found this to be true for acid digests, peptic or pancreatic digests of the same vegetable materials. The size of bacterial colonies on the plant agar is much greater than on meat-infusion agar. Very potent toxins may be produced in these media. When these vegetable media were used as a base for fluid thioglycollate medium, *Clostridium novyi* and *Clostridium tetani* grew out in dilutions as high or higher than in either of the thioglycollate formulae approved by the National Institute of Health as standard sterility test media for biological products. Most of the butyric organisms which fail to grow when veal infusion is used grow on this medium. Water and milk counts made in vegetable agar have been invariably higher than in "standard methods agars". In most instances the numbers of organisms per ml. in the vegetable broth were greater than in infusion broth.

RECOVERY OF AGAR FROM USED MEDIA

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A simple and satisfactory method for the recovery of agar from used culture media has been developed at the Western Regional Research Laboratory. The steps involved are: (1) drying, (2) grinding, (3) washing, and (4) drying.

In the first step the sterilized used culture medium is collected from time to time in a shallow pan which is kept in a drying oven at 60–70°C. or placed in front of a fan. The reaction of the material should be near neutral before it is dried. After several batches have been dried, one after another in the same pan, the crisp mass is scraped into a bottle or can. When a considerable quantity has accumulated, it is ground fine in a Wiley mill containing a 1-mm. sieve.

The ground material is then placed in a glass cylinder containing some water, and tap water is introduced into the bottom of the cylinder through a glass tube. The cylinder, standing in a sink, fills up and overflows. The flow is regulated so that the agar particles are not carried over. A 2-liter graduated cylinder is a suitable washing vessel for 150 to 200 grams of dried agar medium and a glass cylinder 82 mm. x 120 cm. accommodates 600 to 700 grams of the dried medium. Washing is continued until the soluble matter is removed; a flow of 1 liter per 10

minutes for 36 hours usually is sufficient. The agar is filtered on a Whatman No. 4 filter paper in a Büchner funnel and spread out in a one-inch layer to dry. The material dries readily in front of a fan or in a drying oven at 55–60°C.

When new medium is to be made up, the reclaimed agar is melted at 100°C. in the proper amount of water; 10 grams of activated charcoal are added per liter; and the material is filtered while hot through a Whatman No. 3 filter paper on a Büchner funnel with suction. A 15-cm. paper is satisfactory for 1 liter of medium. Beef extract and peptone or other nutrients are added, pH is adjusted, and sterilization carried out as usual.

Additional purification of the agar has been found unnecessary. In the bacteriological analysis of water, milk, tomatoes, and frozen vegetables, the reclaimed agar was in no way inferior to the virgin agar as regards colony development. In comparison with other methods (Edwards, 1942; Macmorine, 1942; Thaller, 1942; Roe, 1942; and Blundell, 1943), the procedure is simple. Nearly all laboratories have the necessary facilities for recovering fairly large batches of agar by the method outlined, which has proved to be neither laborious nor time-consuming. If only small quantities of agar are used, they may be allowed to accumulate, without additional work, to be purified as necessary in large batches. Laboratories would therefore be well advised at least to save their waste agar media according to step No. 1 against a possible critical situation. In any case, this precaution would call for only a trifling amount of time or labor.

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STUDIES ON THE RÔLE OF NIACIN AND THIAMINE IN THE METABOLISM OF GLUCOSE BY STAPHYLOCOCCUS AUREUS

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In a study on the inhibitive effect of glucose, in media devoid of niacin, on the growth of bacteria requiring this vitamin for carbohydrate metabolism, it was noted that there exists a certain coordination between niacin and thiamine in the utilization of glucose by staphylococci. The experiments reported below were designed to elucidate the specific rôles of these two vitamins in the carbohydrate metabolism of these organisms.

Knight, in 1937, was the first to demonstrate that *Staphylococcus aureus* can grow in a synthetic medium containing cystine, tyrosine and tryptophane, provided the two vitamins niacin and thiamine are also added. He designated the vitamins as essential growth factors, but did not establish their specific function in the metabolic activity of these organisms. Subsequent metabolic studies have been made chiefly on resting cells previously grown either in media containing adequate amounts of both vitamins, or, as in the work of Hills (1938), in substrates relatively deficient in thiamine, but not in niacin. The authors referred to were concerned primarily with the nature of the breakdown of pyruvic acid, because of the part played by thiamine in this process in animals.

The present investigations differ essentially from the previous studies in that they were conducted with growing cells and were designed to ascertain the rôle of these vitamins singly and together in the metabolism of glucose and the resulting acids. For comparison, a number of respiration tests were also made with resting cells. Experiments were conducted under both aerobic and anaerobic conditions.

EXPERIMENTAL

Procedure and methods. The staphylococci used in these experiments were isolated from furuncles. For preliminary orientation a large number of freshly isolated and stock strains were tested for their vitamin requirements. Most of the cultures needed both nicotinic acid and thiamine; some strains grew well without the addition of thiamine, but niacin was essential for all of them.

The medium used in these experiments was made up as follows:

A. Salt solution:

NaCl.....	5.0 gm.
Na ₂ HPO ₄	2.5 gm.
KH ₂ PO ₄	0.35 gm.
H ₂ O.....	900 ml.

This solution (pH 7.2) was divided into tubes, 9.0 ml. per tube, and autoclaved. To each tube were added: 0.5 ml. of a 10%, vitamin-free casein hydrolysate and

0.01% tryptophane (destroyed in the acid hydrolysis), or 0.05% indole. To this basic medium, glucose, lactate or pyruvate was added in the amounts indicated from sterile concentrated solutions. Constant amounts of the vitamins (1.0 γ per ml.) were used throughout. In the anaerobic experiments 10 γ , uracil were added to each ml. of medium. The tubes were inoculated with 1000–10,000 washed organisms and incubated in a slanting position at 37°C., for 4–5 days. At the end of the incubation period growth density was determined by a photometer, 0.7 ml. of N/1 H₂SO₄ added to stop further metabolic activity, and the cultures used for chemical analysis.

Anaerobic cultures were grown in a McIntosh apparatus. Since complete anaerobiosis is essential for these tests, two sets of controls were used: the usual sterile medium with methylene blue, and a culture in a lactate medium which grows in the presence of a residue of oxygen but gives no growth under complete anaerobic conditions.

The chemical analyses were made by the following procedures: Glucose by the iodometric method of Lehmann-Maquetenne; lactic acid by the method of Friedmann and Graeser (1933); acetic acid by the Friedmann method (1936). Pyruvic acid was determined by two methods; that of Clift and Cook (1932), and that of Lee (1939), with a slight modification.

Each experiment was repeated several times with fairly consistent results.

RESULTS

Under *aerobic* conditions, the extent and nature of the utilization of glucose, lactate and pyruvate appeared to depend on whether one, both or neither of the vitamins was present.

When neither of the vitamins or only thiamine was present in a medium containing glucose or pyruvate there was practically no growth or utilization of either glucose or pyruvate; under these conditions there was slight oxidation of lactate.

When both vitamins are present the results are totally different. Under these conditions 17 to 20 mg. of glucose is metabolized (the amount used is never higher, whatever the amount of glucose in the medium, due to the limiting acidity). The metabolism proceeds to the final oxidation products, acetic acid and CO₂. The metabolic end products of the glucose used are, lactic acid 20%, acetic and other volatile acids 40%, and pyruvic acid only in very small amounts. In the pyruvate medium about 3.0 mg. of the pyruvate is metabolized and practically all of the amount used is recovered as volatile acid in equivalent amount; only a small amount of lactic acid is formed. It would seem that this is an oxidation reaction as suggested by Barron and Lyman (1939). In the lactate medium, in the presence of both vitamins, about 10 mg. of lactic acid are metabolized with the formation of an equivalent amount of acetic acid; this is clearly an aerobic oxidation.

When only nicotinic acid (without thiamine) is present in the substrate, much less of the available substance is utilized and the oxidation is incomplete. Under these conditions, only 6–7 mg. of glucose are used (about 37% of the amount used when both vitamins are present). Of the glucose used about 65% appears as lactic and 40% as pyruvic acid; the reaction approximates that of simple glycoly-

sis. This accumulation of pyruvic acid is similar to that noted by Peters (1936), in the brains of pigeons suffering from B₁ (thiamine), avitaminosis. Unpublished studies in this laboratory have shown that within certain limits the disappearance of the pyruvic acid is directly proportional to the concentration of thiamine added and can serve as a quantitative test for this vitamin. In the pyruvate niacin medium only about half as much pyruvate is utilized as when both vitamins are available. About 30% of the pyruvate appears in the form of lactic acid and the rest as volatile acids. The utilization of lactic acid is half the amount metabolized when both vitamins are available and an equivalent of 25% of the amount used accumulates as pyruvic acid; in this case too, the oxidation is incomplete.

It is apparent then that nicotinic acid is essential for glycolysis as well as for the oxidation of lactic acid. If only this vitamin is present, the carbohydrate utilization is limited and partial, with the accumulation of pyruvic acid. The complete oxidation of lactic and particularly pyruvic acid to volatile and acetic acids occurs only when thiamine is also present. The function of thiamine under aerobic conditions is, therefore, that of catalyzer of oxidation and supplementary to that of nicotinic acid.

In a carbohydrate-free medium the results are the same as in the lactate and pyruvate media, indicating a similar process. It seems that the amino-acids in the medium either break down into these acids, or substances closely related to them. Thus, in the presence of both vitamins an appreciable amount of "volatile acids" is formed, while in the absence of thiamine, there is an accumulation of a substance which gives all the reactions of pyruvic acid. These results account for the favorable effect of these vitamins on growth in an amino-acid medium without carbohydrates.

Under *anaerobic* conditions the results are distinctive. There is no growth in the lactate medium even if both vitamins and uracil are added. Neither is there growth in the carbohydrate-free medium.

In the glucose and pyruvate media the results depend entirely on the presence or absence of niacin. Under anaerobic conditions thiamine has no effect on glucose metabolism. If thiamine is present and niacin is absent none of the sugar is used and there is no growth. Practically the same results are obtained when niacin alone is present as when both vitamins are present. Pyruvic acid is not formed from glucose; the principal product (65–70%) is lactic acid (1.4 mol lactic acid to 1 mol glucose). The reaction is purely glycolytic. In the pyruvate medium an equivalent amount of lactic acid is formed (2 ml. pyruvic acid = 1 mol lactic acid). This appears to be a dismutation reaction: 2 pyruvic acid + H₂O = lactic acid + acetic acid + CO₂.

Because these results differ from those reported by other authors the experiments were repeated several times with essentially the same findings. It seems that, at least insofar as this test organism is concerned, thiamine has no effect on glucose or pyruvate dismutation under anaerobic conditions.

The logical deductions from these experiments is that thiamine is concerned only in the oxidation and not in the dismutation reaction. If that is correct the results obtained under aerobic conditions can be brought into harmony with those noted in the anaerobic environment. In the presence of oxygen and ab-

sence of thiamine, pyruvate is broken down by dismutation according to the reaction of Krebs (1937); lack of absolute agreement in the analytical data is due to partial oxidation of the lactic acid formed, under aerobic conditions. When both niacin and thiamine are present both dismutation and oxidation occur. It is conceivable that the variable results obtained by other workers are due to the differences in the relative amounts of the two vitamins present in the substrate.

Respiration experiments. To complete the picture, experiments were designed to ascertain the effect of these vitamins on resting cells. Because of the difficulty of obtaining a sufficiently large quantity of cells in media free of vitamins, cultures had to be grown in a substrate containing only nicotinic acid and the effect of thiamine on such cells tested.

The procedure was that employed in previous experiments (Kligler and Grossowitch, 1941). Cultures were grown in 250 ml. flasks containing 100 ml. of the medium described above with the addition of 0.1% lactate and 1.0 γ , nicotinic acid per ml. After 48 hours incubation the cells were collected by centrifugation and washing and a heavy saline suspension prepared, giving a reading of 15 on the photometer. This was the stock suspension used in the respiration experiments.

The actual respiration tests were made in ordinary pyrex test tubes specially cleaned and prepared for this purpose. Five ml. of the reacting substances and cells were added to the tubes and the fluid covered with 5 ml. of hot vaseline which was then cooled quickly and placed in a 30°C. water bath. Frequent readings were taken and the minimum time of complete reduction of the methylene blue indicated the relative rate of respiration.

The "system" as set up was as follows:

M/15 phosphate buffer (pH 7.2)	0.5 ml
1/7000 methylene blue	0.5 ml
Bacterial suspension	0.5 ml
M/20 solution of glucose, lactate or pyruvate.	0.5 ml
Distilled water	3.0 ml
Thiamine hydrochloride 1 γ /ml.	

Controls contained all substances except the donator.

The results of a typical experiment are summarized below:

Time of reduction of M.b. in system (minutes)

DONATOR	WITH THIAMINE	WITHOUT THIAMINE	ACCELERATION DUE TO THIAMINE
Glucose	8	31	ca. 4.0 times
Pyruvate	11	28	ca. 2.5 times
Lactate	11	21	ca. 2.0 times
None	27	147	

Thiamine has the greatest accelerating effect on the oxidation of glucose, and least in that of lactic acid. These results correspond with those obtained with growing cells.

SUMMARY

Nicotinic acid is the essential factor in the glucose metabolism of the strains of *Staphylococcus aureus* studied; without it there is no fermentation and only slight growth.

If niacin alone is present and thiamine lacking there is active growth as well as partial utilization of available carbohydrates. Under aerobic conditions, the amount of glucose metabolized is about 40% of that used when both vitamins are present. About 40% of the glucose consumed is converted into pyruvic acid and 60% into lactic acid, indicating that in the absence of thiamine the reaction is essentially glycolytic.

When both vitamins are present, two and a half times as much glucose is utilized as when niacin alone is present and the end products consist of about 40% acetic acid, 20% lactic acid and only slight amounts of pyruvic acid. It appears, therefore, that thiamine acts as a catalyzer in the oxidation of pyruvic acid and can only exercise this function when nicotinic acid is available for the primary glycolysis.

Under *anaerobic* conditions, thiamine apparently has no influence on the reaction, which is glycolytic. Whether both vitamins are present or only niacin, practically the same amount of glucose is used with the equivalent production of lactic acid.

When pyruvate is used in place of glucose, the absence of thiamine results in dismutation according to the reaction of Krebs. This is very clear under *anaerobic* conditions when the equivalent amount of lactic acid is formed irrespective of whether thiamine is present or not; under aerobic conditions this reaction is obscured to some extent because some of the lactic acid formed is apparently oxidized.

Lactate in a substrate is used only under *aerobic* conditions. In the presence of both vitamins the oxidation is complete to acetic acid and CO₂. In the absence of thiamine the oxidation is incomplete with a certain accumulation of pyruvic acid (about 25% of the lactic acid used).

Respiration experiments with resting cells indicate that thiamine accelerates the oxidation—of glucose 4 times, of pyruvic acid 2.5 times and of lactic acid twice. These results are in line with those obtained with growing cells.

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SELECTING, INBREEDING, RECOMBINING, AND HYBRIDIZING COMMERCIAL YEASTS¹

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A BREEDING PROGRAM

The first step in a breeding program is the selection of strains with different useful characteristics. A few of the specific requirements of a good bakers' yeast generally recognized by commercial producers are:

- (1) Keeping quality. A good yeast does not autolyze nor lose viability excessively when held in a cake for a few days at room temperature.
- (2) Baking strength. A good yeast causes dough to rise quickly.
- (3) High yield. A good yeast transforms the maximum amount of nutrients in the fermentors into yeast in the shortest possible time.

Different strains of bakers' yeast vary in these and other properties.

The second step in a breeding program is the intensive inbreeding of each of the chosen strains. Successful inbreeding accomplishes two results: (1) it intensifies the desirable character, (2) it eliminates undesirable recessive genes and makes the breeding stock homozygous for the desirable traits. Some good breeding stocks do not give outward manifestations of their excellence because the inbreeding process which has intensified their vigor in one character may have reduced their vigor in other respects.

The third step is the combination of the different desirable qualities of various stocks by hybridizing. The results of hybridizing are not predictable. One reason is that inbreeding operates at random in the selection of modifying genes. Another reason is that certain characters may be mutually exclusive. For example, it is possible that high yield may result from a physiological mechanism that reduces baking strength, and a given stock may not be able to excel in both characteristics. In the most favorable condition the hybrid may be superior to the two parents in the traits for which the parents were selected; in the most unfavorable condition the hybrid may be inferior to both parents in the respective traits.

In yeasts, the hybrid can be propagated asexually. This means that the problem is simpler than in the higher plants such as maize, for in maize each generation is produced sexually and the maize hybrid deteriorates on propagation due to the segregation of the characters combined in the hybrid. In yeast propagation, sexual reproduction ordinarily occurs only under special conditions and it should be possible to maintain the hybrid combination indefinitely by asexual vegetative propagation.

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HETEROTHALLISM IN *SACCHAROMYCES CEREVISIAE*

The brilliant researches of Winge (1935) and Winge and Laustsen (1937, 1938, 1939a, and 1939b, 1940) proved (1) the existence of a sexual cycle and (2) the possibility of hybridization of commercial yeasts. The present paper confirms many of their findings and lays the basis for a breeding program. They produced many hybrids between sporulating yeasts, but did not outline a successful breeding program, largely because they concluded that *Saccharomyces cerevisiae* was not heterothallic. Winge states, "In these yeasts fertilization does not consist in a union of cells that differ genotypically nor do the cells have to differ phenotypically. It is not a question of + and - cells. . . ." Our conclusions do not agree with this statement. We have found that *S. cerevisiae* is heterothallic and that *legitimate* copulations only occur between genetically different gametes. We have been able to show that the mating types are differentiated by the simple + - allelism rather than by one of the more complex types resembling those found in the smuts, paramecia, and hymenomycetes. Winge is correct in his statement that union of cells can occur between gametes that are identical genotypically but we have concluded that these copulations are *illegitimate*, because they do not regularly lead to the production of viable ascospores.

The facts are shown diagrammatically in figure 2. From a sporulating culture like that shown in figure 1, a single 4-spored ascus is selected. The four spores for this ascus are separated with a micromanipulator and grown separately. Winge showed that haploid and diploid cells can be distinguished morphologically and that a single ascospore germinating alone, produces small, round haploid cells. Copulations between haploid cells produce ellipsoidal diploid cells. We have confirmed these facts by many observations of the germination of single ascospores. In hanging drop cultures arising from a single ascospore, a large colony of haploid cells is usually produced. Such cultures were shown by Winge and Laustsen (1937, Plate II, fig. 6 and Plate IV, fig. 15). The colony of haploid cells arising from a single ascospore may become rather large before diploid cells appear and many of the colonies die before any diploid cells appear. Copulations are often long delayed and the diploid cells are usually found at the edge of the large haploid colony or later, after the original colony has been transferred. When an intact 4-spored ascus is planted on a hanging drop of agar and allowed to germinate, diploid cells appear much more rapidly than in single ascospore isolations. Figures 4 to 15 and Plates I, II, and III in Winge's 1935 paper show clearly that when the 4 spores of an ascus germinate together, there is little or no delay in copulation. This is in agreement with our observations on the germination of numerous intact 4-spored asci. The marked difference between microcolonies arising from a single ascospore and a single ascus supports the view that *S. cerevisiae* is heterothallic. All the haploid cells in a young, single ascospore culture are genetically identical and, therefore, of the same mating type; the tendency to copulate is rather low and copulation is delayed. When 4 spores are formed in an ascus, the reduction division segregates the mating type alleles and produces spores of different mating types. When the

intact ascus with its 4 spores germinates, each spore produces haploid gametes of the mating type corresponding to the spore from which it originated. These gametes are all crowded together in a single small colony and since two or more mating types are available, copulations occur immediately.

ASCOSPORES FROM SINGLE ASCOSPORE AND SINGLE ASCUS CULTURES

Winge further stated: "A further proof that there is no sex differentiation in these fungi, and no + and - spores, may be seen in the fact that single



FIG. 1. *Saccharomyces cerevisiae*.—A CONSPICUOUS 4 SPORED ASCUS WITH 3-, 2-, AND 1-SPORED ASCI AND VEGETATIVE CELLS.

Mixtures of this type are the rule. The material has been transferred from gypsum spores which have been isolated and germinated can give origin to cultures which are able to produce asci and spores quite normally."

We have made critical examinations of several hundred single ascospore cultures. Some single ascospore cultures produce no asci at all, others produce fewer than the legitimate cultures and rarely one is found which sporulates abundantly. Moreover, the viability of the ascospores from single ascospore

cultures is always extremely low, and for this reason the spores cannot be said to have been produced "normally." Four-spored asci are generally much rarer in single ascospore cultures than in legitimately diploid cultures.

In single ascospore colonies, all the haploid cells are identical, and only illegitimate copulations can occur. These illegitimately diploid cells are generally incapable of producing viable 4-spored asci and the single ascospores isolated from these cultures are even less capable of growth in culture than single ascospores isolated from the parent culture. Winge has called this phenomenon inbreeding degeneration, but this implies degeneration due to randomly distributed dysgenic factors. It appears from the preceding discussion that it is

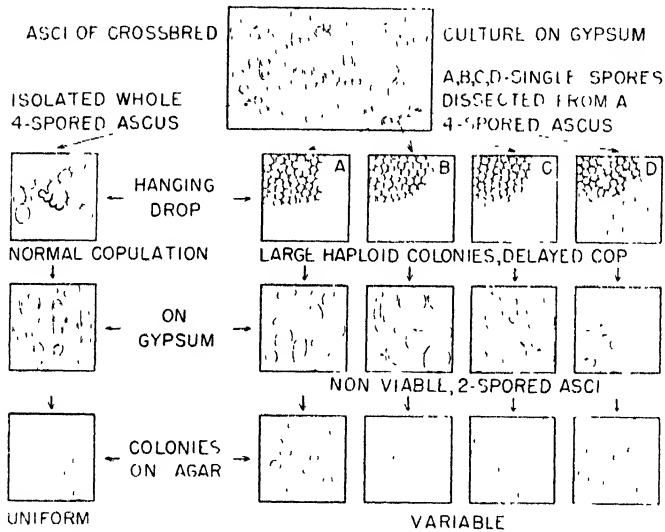


FIG. 2. DIAGRAM SHOWING THE BASIS FOR CONCLUDING THAT *N. crassa* IS HETEROTHALIC

Intact 4-spored ascus produces diploid cells almost immediately. Isolated single ascospores produce micro-colonies containing numerous haploid cells, copulation is delayed and diploid cells appear later. Four-spored asci are much rarer on gypsum from single ascospore colonies than from intact ascus colonies; many single ascospore cultures do not sporulate at all. Colonies that appear when cultures are plated on agar show uniformity in the case of intact asci, but great variability in the case of the single ascospore cultures. The ascospores produced by single ascospore cultures are generally non-viable while the ascospores from intact ascus cultures are highly viable.

due rather to homozygosis for the mating type alleles which are functionally identical with self-sterility genes and to those genes which we previously called "sex" genes in *Neurospora*. Jennings (1910) has introduced the term "mating type" as a result of his work on *Paramecium* and this seems to be the term of choice. Legitimately diploid cultures, heterozygous for the complementary mating type alleles, are distinguished principally by their capacity to produce viable 4-spored asci. We have made numerous observations which prove that only the zygotes from self-fertilized asci (or from hybrids) are able to produce "normal" asci. Normal asci contain four spores, with a high percentage of viability.

The above conclusion that homozygosis of the mating type alleles is the primary cause of the low viability of the ascospores from single ascospore cultures (segregation of recessive genes independently gives rise to dark color, rough colony, low yield, and poor production of ascospores) does not necessarily invalidate Winge's view concerning the possibility that an additional effect on low viability may be due to some cytoplasmic component. Winge recognized the reduced viability of ascospores arising from single ascospore cultures in which the zygote is produced by the copulation of two of the haploid gametes grown from the original single spore. He discovered that this low viability was further reduced in cultures arising from zygotes produced by the direct diploid germination of a single spore (which presumably contained two haploid nuclei). He concluded that this was proof of a cytoplasmic effect.

It is possible to maintain sporulating cultures in the laboratory. This is done by self-fertilizing normal 4-spored asci. The procedure is simply to allow an intact 4-spored ascus to germinate in a hanging drop. When a colony has formed, it is plated on agar and single colonies are tested for their ability to produce 4-spored asci on gypsum. Only some of the colonies selected from the self-fertilized, intact 4-spored ascus cultures are capable of producing 4-spored asci which suggests that some illegitimate copulations also occur under these conditions. However, the only cultures in all our several hundred isolations which were capable of producing vigorously viable 4-spored asci were from self-fertilized asci or hybrids and this is the exclusive source of normal asci and ascospores. This strongly supports the view that normal ascospores are only produced by a diploid culture in which the cells are heterozygous for the mating type alleles. These zygotes are produced by a legitimate copulation, i.e., one which occurs between two genetically different spores (or gametes) of complementary mating types.

VARIATION AMONG THE COLONIES GROWN FROM SINGLE ASCOSPORE AND SINGLE ASCUS CULTURES

The colony grown from an intact 4-spored ascus is generally of the wild-type and this is invariably true if it produces an abundance of viable 4-spored asci, that is, if it is the product of a legitimate copulation. When the four spores are dissected from an ascus and an individual colony grown from each, these four colonies are generally variants from wild-type. Winge showed that each of these colonies generally has a characteristic phenotype which can be referred to segregation at the reduction division which preceded spore formation.

When the wild-type colonies obtained by the self-fertilization of an intact 4-spored ascus are subcultured by plating on agar, uniform large smooth colonies are obtained. However, when the variant colonies obtained from the single ascospore cultures are similarly plated, a number of new different variants appear. An experimental study of this phenomenon is in progress. Storage of a single ascospore culture increases the variation between the colonies grown from it because mutations occur during storage. Storage also increases the variability of the cultures from intact asci presumably for the same reason.

When a single 4-spored ascus germinates on the hanging agar drop, the haploid cells copulate with each other apparently at random, although this is extremely difficult to follow. Presumably some cross-bred zygotes are produced. In our accounting system we designate the four ascospores from one ascus arbitrarily as A, B, C, and D. There are ten possible copulations as follows:

A × A	A × B	B × C
B × B	A × C	B × D
C × C	A × D	D × D
D × D		

The four homozygous copulations (A × A, B × B, C × C, and D × D) probably occur rather infrequently judging from the fact that in colonies produced by single ascospores, diploid cells occur much less frequently and much later than in colonies originating from a whole 4-spored ascus. Since two mating types exist in *S. cerevisiae*, (+) and (−), one possibility is A+, B+, C−, and D−. This permits four legitimate matings: A+ × C−, A+ × D−, B+ × C−, B+ × D−. The fact that the ascospores are not all viable, places a further limitation on the number of legitimate copulations which may occur. If only one spore survives per ascus, the culture originating from a single 4-spored ascus will have all the characteristics of a single ascospore culture. If two genetically identical spores survive, copulation should likewise produce a culture indistinguishable from a single ascospore culture. However, whole 4-spored asci produce viable cultures more frequently than would be expected on the basis of the data obtained from the isolation of single ascospores. This suggests that two haploid cells which would die if isolated, may produce a diploid cell capable of indefinitely continued multiplication. We define viability of ascospores in the same manner as Winge, namely, if (1) an ascospore fails to germinate or (2) grows to produce a small haploid colony of cells which is not capable of indefinitely continued growth in culture, the ascospore is considered non-viable. Gametes from an isolated ascospore not permitted to copulate with a genetically complementary mating type may be generally weak and usually incapable of producing a colony which can multiply indefinitely in culture, although they may be capable of copulation with gametes of a complementary mating type if such are available. A haploid colony unable to persist when isolated, might be capable of producing a vigorous diploid culture after copulation with cells from an equally weak, similarly isolated, haploid colony. There is a parallel in the case of the smuts where haploid cultures can usually multiply indefinitely in culture, but are incapable of acting as parasites except after copulation with other haploid cells to produce pathogenic diploid cells. Only diploid or dikaryotic cells are capable of growing in the host plant, but they are only produced by copulation between two gametes of appropriate complementary mating types.

In *S. cerevisiae* only one-half (Lindgren and Lindgren, unpublished) to two-thirds (Winge and Laustsen, 1940) of the ascospores are capable of producing cultures which can multiply indefinitely in culture. This does not mean, how-

ever, that the remainder of the ascospores are non-viable in the strict sense of the word nor that conventional lethal factors are involved.

FIRST GENERATION

WILD TYPE YEAST "X", YIELD 6.5, WHITE, SMOOTH, LARGE COLONIES

4-SPORED ASCUS SELF FERTILIZED
ASCUS 1

Some zygotes heterozygous for mating type alleles. Practically no haploid cells persist in culture. Copulation not delayed.

4-SPORED ASCUS DISSECTED
ASCUS 2

4 single ascospore cultures. Diploid cells homozygous for mating type alleles, together with haploid cells.

COLONIES GENERALLY UNIFORM,
LARGE SMOOTH COLONIES TESTED

plated
←



Ascus Colony	4-spored asc	Yield
X1 a	none	5.0
X1 b	none	5.0
X1 c	none	5.5
X1 d	none	6.5
X1 e	none	6.0
X1 f	present	6.5
X1 g	present	6.0

	X2A	X2B	X2C	X2D
YIELD	5.5	5.0	4.5	3.0
COLOR	white	white	dark	dark
COLONY	smooth	smooth	rough	rough

SECOND GENERATION

X1g

4-SPORED ASCUS SELF FERTILIZED
ASCUS 1

LARGE UNIFORM COLONIES; COLONIES TESTED

ASCUS COLONY	4-SPORED ASCI	YIELD
X1g 1 a	none	5.5
X1g 1 b	none	6.0
X1g 1 c	none	6.0
X1g 1 d	none	6.0
X1g 1 e	present	6.0
X1g 1 f	present	6.5
X1g 1 g	present	6.5

4-SPORED ASCUS DISSECTED
ASCUS 2

FOUR SINGLE ASCOSPORE CULTURES

X1g 2A X1g 2B X1g 2C X1g 2D

	X1g 2A	X1g 2B	X1g 2C	X1g 2D
Yield	5.5	5.0	5.0	5.5
Color	white	white	white	white
Colony	smooth	smooth	smooth	smooth

This analysis shows culture X1g is homozygous for wild type dominant genes. Recessives differentiating low yield, dark color, and rough colony have been bred out. These single ascospore cultures cannot form viable ascospores and therefore cannot be used for matings.

THIRD GENERATION

X1g 1g

4-SPORED ASCUS DISSECTED, ASCUS 1

SINGLE ASCOSPORE CULTURES TESTED

	X1g 1g 1A	X1g 1g 1B	X1g 1g 1C	X1g 1g 1D
Yield	5.5	5.5	5.5	5.0
Color	white	white	white	white
Colony	smooth	smooth	smooth	smooth

This analysis shows that culture X1g 1g produces viable ascospores and is homozygous for wild type alleles. Therefore single ascospores from it can be used as gametes.

FIG. 3. PEDIGREE SHOWING THREE GENERATIONS OF YEAST SELECTION AND INBREEDING

INBREEDING PROCEDURES

Yeasts can be inbred by mating closely-related ascospores of complementary mating type. This insures the continued ability to produce the viable 4-spored asci essential to a breeding program. Our diagram (fig. 3) shows a synthetic pedigree illustrating our routine laboratory procedure. A single 4-spored ascus from culture "X" is planted on a hanging drop of agar and the colony is per-

mitted to grow to full size. The entire colony is transferred to a stock culture tube and later plated on agar. Single colonies are tested for yield. Selected single high-yielding sub-cultures from the plates are transferred to an appropriate medium and later to gypsum slants on which ascospores are produced. They vary considerably in their ability to produce 4-spored asci. The best culture, in regard to the production of ascospores, high yield, and capacity to ferment a variety of sugars, or such other desirable characters as can be easily tested, is selected for the next generation; from the sample pedigree, X1g is the culture of choice from the first generation.

In addition to self-fertilized asci, other asci from the "X" culture are isolated and individual ascospores dissected from them with a micromanipulator and grown separately. Study of these cultures reveals the degree of heterozygosis of the original culture. Most commercial yeasts are heterozygous. Single ascospore cultures from 4-spored asci of a smooth colony form often produce some rough type cultures, in the first generation. Both rough and dark yeasts are inferior baking yeasts and the genes differentiating these characters are recessive. Since the inferior genes are usually recessive, they can only be detected by growing single ascospore cultures from 4-spored asci. The fact that some of the single ascospore cultures are rough, dark, small-colonied, or produce excessively low yielding cultures, proves that the original culture was heterozygous. Analysis of ascus 2 in the same pedigree proves that the "X" yeast is heterozygous for recessive genes for roughness, and dark color; low yield is usually associated with these characters, especially with dark color.

Continued inbreeding can produce a homozygous strain and eliminate the inferior genetic material. Inbreeding and selection are continued until none of the four single ascospore cultures from a single ascus produces any distinctly inferior cultures, proving that the strain no longer carries inferior recessive genes. Inbreeding is not continued until single ascospore cultures are obtained which give the same yield as the cultures heterozygous for the mating type alleles. The yield of single ascospore cultures seems to be generally lower than that of many of the legitimately diploid cultures, possibly due to recessive mutations closely linked to the mating type locus.

In practice, 8 or 10 single 4-spored asci are dissected and a similar number are self-fertilized in each generation. The second generation originates from one of the selected cultures from a self-fertilized ascus which produces abundant viable 4-spored asci. If selected 4-spored asci are self-fertilized for several inbred generations, cultures can eventually be found which produce more ascospores than the original. We have inbred several strains by the serial selection of whole 4-spored asci for five generations without any diminution of ascospore production. This is proof that inbreeding does not produce degeneration which results in the loss of ability to produce ascospores. Single ascospore cultures from the later generations resemble those from the first generation in their inability to produce 4-spored asci. The single colonies obtained in the second generation by plating the culture grown from a self-fertilized intact 4-spored ascus are individually tested for yield and then subsequently placed on gypsum

to test their ability to produce 4-spored asci. They are not all capable of producing 4-spored asci. Many of them resemble single ascospore cultures. This fact suggests that some illegitimate copulations occur in selfing. The sub-cultures which produce 4-spored asci and which yield well are selected for the third generation.

The single smooth, white culture "X1g" selected to give rise to the second generation will be homozygous for smooth-colony, white-color genes, if it originated by copulation between two gametes neither of which carry recessive genes. This can be tested by the dissection of a 4-spored ascus from the second generation. In practice many dissections must be made before an ascus is discovered from which all four spores survive and each produces a culture, but a single test ascus of this type is sufficient to prove the absence of recessive genes in the parent culture. In the sample pedigree ascus X1g2 produced four single ascospore cultures showing the phenotypes of the dominant genes, which proves that culture X1g is homozygous for the dominant alleles and the recessive genes have been bred out of the stock. This test also shows that the ascospores are viable; this culture can be used as a breeding stock.

A similar test in the third generation shows that culture X1g1g is likewise available as a breeding stock, presumably very similar to X1g.

RECOMBINATION VERSUS INBREEDING

Quantitative characters such as yield and baking strength usually depend on the interaction of a large number of genes, just as a good hand in cards depends on the correlated support of a large number of cards. An inbreeding program which purifies the stock of clearly undesirable genes has the possible disadvantage of resulting in the irretrievable loss of some members of the gene complex essential to the full expression of the quantitative character. While inbreeding seems the preferred method of approach to developing a breeding stock from a relatively poor heterozygous strain containing a large number of clearly deleterious genes, another method has given us better results with good yeasts carrying only a few deleterious genes. This is the method of producing recombinations in the first generation. Making all selections from the first generation prevents the irretrievable loss of minor (recessive or partially dominant) genes which are not easily detectable but which contribute cumulatively to the value of the yeast as a deuce contributes to the strength of a run of trumps.

The reduction divisions preceding spore formation shuffle the yeast genes by two mechanisms (1) random assortment of chromosomes and (2) crossing-over (i.e. the breakage and reunion of chromosomes). Genes are not good or bad *per se*, but only produce their characters with the cooperation of the genetic environment in which they exist and the character which a gene exhibits against a changed genetic background is unpredictable. This is consistent with what we have found in testing diploid strains obtained by self-fertilization from the first generation of a good yeast containing only a few clearly deleterious genes. The original culture had a rather high yield but poor baking strength. Single

Figure 4 illustrates the procedure by which two spores from different strains are paired. In fig. 5, photographs of the 4 colonies grown from four pairs of spores are shown. We designate the hybrids as follows: $A \times A$, α ; $B \times B$, β ; $C \times C$, γ , and $D \times D$, δ . Colonies α and β are both haploid. This suggests that the two spores were not of complementary mating types. Colony γ contains many diploid cells suggesting that a hybrid may have been produced. The true hybrid produced by legitimate copulation can be distinguished by its ability to produce viable 4-spored asci. This is tested by transferring the cultures arising from the individual colonies on the agar plate to gypsum. If the two paired ascospores are not of complementary mating types, the hybrid will be illegitimate and will not be capable of yielding viable ascospores and will not be available for further breeding experiments.

A second method of hybridization was attempted. This was to mate one intact 4-spored ascus from one breeding parent with another 4-spored ascus from the other breeding parent. It was thought that this might assure the presence, in one small colony, of gametes from both breeding parents of appropriate mating types. The yield of hybrids by this method was quite low, presumably because the four spores of each intact ascus tend to copulate *intra-se* with the production of only a few haploid cells, thus limiting markedly the amount of copulation which occurs among all eight spores *inter se*.

THE ORIGIN OF CULTIVATED YEASTS

Domesticated plants and animals are specialized, usually at the expense of characters which make for survival under conditions of natural competition. Milk cows cannot survive in the wild state, for survival value has been reduced in the development of milk cows to promote milk production. Corn seeds are accidentally sown by the hundreds of thousands, but corn plants are practically never found on the wayside nor in any place except a cultivated field for the domesticated plant is incapable of competing with wild plants under natural conditions. Wild plants are in genetical balance with the environment. Domestication disturbs this balance and produces specialized variants in which the characters essential to competition in the natural state have been diminished in potency. These facts also apply to the domestication of yeasts. Most cultivated yeasts have probably originated from wild yeasts by the process of spore formation, illegitimate copulation and mutation. Wild yeasts are probably heterozygous for genes capable of increasing or decreasing the CO_2 production from the optimal efficient amount consistent with growth under natural conditions to amounts either larger or smaller than normal. These genes would be inefficient under natural conditions but may be desirable in some domesticated yeasts. Reduction division, spore formation and illegitimate copulation could produce diploid cells homozygous for either the plus or minus modifiers. Wild yeasts probably contain plus and minus modifiers capable of affecting practically all the genes producing enzymes. For example if sugar is transformed into CO_2 by the production of intermediate products such as pyruvic acid, acetone, acetaldehyde, and alcohol, plus or minus modifiers could increase or diminish

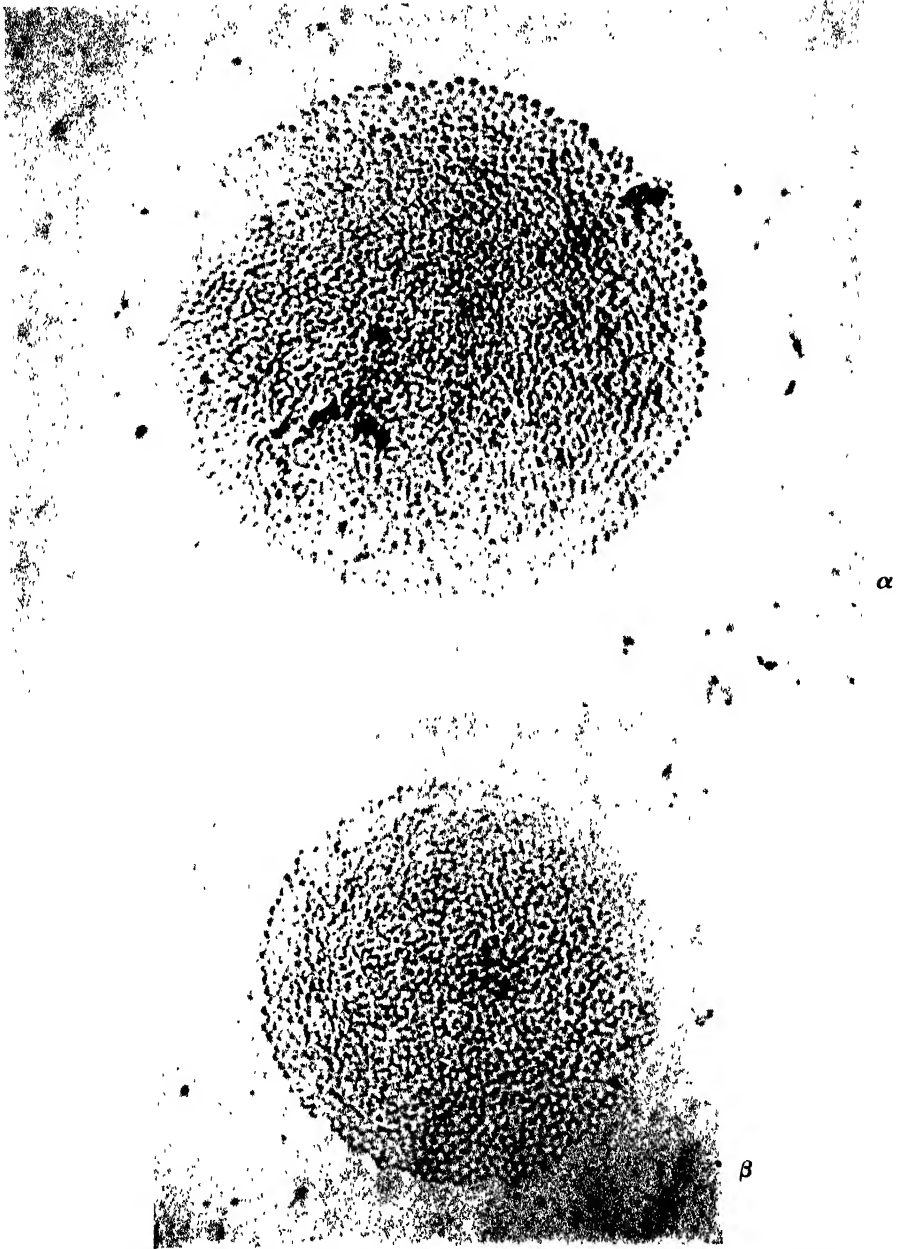


FIG. 5. PHOTOGRAPHS OF THE COLONIES PRODUCED BY THE MATINGS DIAGRAMMED IN FIGURE 4

Colony α has been produced by mating an A ascospore from the X line by an A ascospore from the Y line. It is a large colony containing only haploid cells. No hybrid has been produced. This colony may be a mixture of two kinds of haploid cells or only one of the ascospores may have germinated. Colony β also contains only haploid cells. Colony γ contains diploid cells. These may be hybrid or they may have been produced by illegitimate copulation. Colony δ contains only haploid cells and has grown rather poorly.

the amounts of each of these intermediate products. Most bakers', brewers' and distillers' yeasts which we have examined are illegitimately diploid strains. They were probably produced from wild yeasts by the chance selection of single



FIG. 5

ascospore cultures homozygous for some plus modifier of a gene affecting some chemical stage of the fermentation process.

Illegitimately diploid cultures are usually definitely inferior to the normal diploid type. This is shown by the low yield and poor viability of most single

ascospore cultures obtained in the first generation from heterozygous yeasts. This may be due to the segregation of recessive genes closely linked to the mating type alleles.

THE NATURAL FUNCTION OF THE SYSTEM OF MATING TYPE ALLELES

The mating type alleles or self-sterility genes are functionally identical with what we have called the sex genes in *Neurospora*. In yeasts, this apparatus serves the same function which it serves in other plants. It insures cross breeding and limits the amount of inbreeding which may occur. In the natural state, yeasts develop vegetatively principally during the late summer and early fall when fruits ripen. It is chiefly the spores which survive the cold winter and dry summer months in the soil. Since only the cross bred or legitimately diploid strains produce viable ascospores, it is only these forms which survive and germinate to grow vegetatively on the ripe, bursting fruits.

Cross breeding maintains the wild type character and reduces the production of recessive types. If the recessive mutations in one strain of yeast are different from those in a second strain all hybrids will be wild type. For the reasons indicated, freshly isolated cultures are usually capable of sporulating abundantly. Cultures carried in the laboratory often lose their ability to sporulate, because of the accidental isolation of single ascospores or of diploid cells produced by illegitimate copulation.

SUMMARY

A breeding program for *Saccharomyces cerevisiae* must be based on the maintenance, under laboratory conditions, of strains producing viable ascospores, since the ascospores produce the gametes. The strains can be maintained if each generation is cross-bred, for the ability to produce viable ascospores depends on the maintenance of heterozygosis of the mating-type alleles, or self-sterility genes. Selection and inbreeding eliminate undesirable recessive genes from the strain. Breeding strains from which these undesirable recessives have been eliminated can be used in hybridization experiments. This technique of improving commercial yeasts differs radically from previous methods which were probably based on the accidental selection of single ascospore cultures from wild yeasts.

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MICROBIOLOGICAL ASPECTS OF PENICILLIN

III. PRODUCTION OF PENICILLIN IN SURFACE CULTURES OF *PENICILLIUM NOTATUM*

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Steadily increasing interest in penicillin as a chemotherapeutic agent has led to a parallel increase in the demand for penicillin for clinical application and chemical studies. Penicillin is produced in minute quantities by *Penicillium notatum* so that filtrates from the mold represent exceedingly dilute solutions of penicillin from which the active agent is extracted. The magnitude of the problem may be illustrated by the experience of the Oxford workers (Abraham, *et al.*, 1941). From 3 to 5 grams of crude penicillin which, on the basis of a later publication (Abraham, Chain and Holiday, 1942), could be at best only approximately 5 per cent pure, was required for a single clinical treatment, such an amount requiring the extraction of 300 to 500 liters of *P. notatum* filtrate. The extreme lability of penicillin lends additional complexities to the production of the active substance.

Aside from the report by the Oxford investigators (Abraham, *et al.*, 1941), little information pertaining directly to the microbiological aspects of penicillin production has appeared (see also Challinor, 1942; Clutterbuck, *et al.*, 1932; Fleming, 1929, 1932; Foster and Wilker, 1943; Foster and Woodruff, 1943; Hobby, *et al.*, 1942; Kocholaty, 1942; Reid, 1935). This paper presents some general characteristics of the production of penicillin and their relation to the metabolism of *P. notatum*. Estimation of penicillin potencies were for the most part made by the modified Oxford cup method (Foster and Woodruff, 1943). In a few cases the dilution method was used.

EXPERIMENTAL

Strain selection

While the nature of the medium has an important influence on maximum penicillin formation, the relative capacity of strains to produce penicillin can be evaluated by routine comparisons on one or a few suitable media (see below). Chances for improvements in the penicillin content of cultures through modifications in the medium and cultural conditions probably are not as great as those which can be effected at the beginning by working with the best strain of *P. notatum* obtainable.

Selection of highest potency strains is of first importance for maximum penicillin activity. Strains of *P. notatum* from numerous different sources differ widely in penicillin activity. Parallel testing in a medium favorable for penicillin production identifies the best strain, and this is selected for the spore-

preservation procedure outlined below. Single assays are insufficient to evaluate a strain, and daily assays on cultures over a period of 5 to 14 days may be necessary. Experience has shown that results obtained from single treatments have very little quantitative significance; triplicate treatments are almost essential.

Degeneration of penicillin-producing ability of strains of P. notatum; preservation of active cultures

Cultures of *P. notatum* tend to lose spontaneously their ability to form penicillin, either entirely or partially. This behavior is not confined to penicillin formation by *P. notatum* but is the "physiological" or "biochemical degeneration" well known for microorganisms. Clutterbuck, Lovell and Raistrick (1932) have already noted this variability or degeneration of *P. notatum* in connection with the formation of the yellow pigment, chrysogenin, and also penicillin. The penicillin-producing capacity of *P. notatum* seems particularly susceptible to degeneration, which occurs quite rapidly if rigorous control is not exercised. It may take place without any conspicuous cultural changes, although frequently degenerated cultures show a reduction in the tendency to sporulate abundantly. An indication that a culture is apt suddenly to lose or be weakened in its ability to produce penicillin may frequently be observed in spore cultures of the mold, especially on agar surfaces. Cultures allowed to incubate a few days beyond the time of maximum sporulation begin to show isolated secondary developments. At first small, white, cottony patches, they increase and gradually spread over the surface. Such cultures are most apt to give sub-cultures weak in penicillin activity.

Continued successive transfers on artificial media are conducive to degeneration of *P. notatum* and of microorganisms in general. Two possible interpretations exist: 1) Cells which have high penicillin-producing capacity give rise to daughter cells (through spores or vegetative cells) which progressively lose this capacity. 2) Each culture is composed of a mixture of substrains or cells heterogenous in regard to this capacity, and during continued propagation the poor substrains overgrow the good strains and predominate in the culture. The good strains in the mixture cannot thereafter compete effectively for nutrients, and the culture as a whole has a reduced capacity for penicillin formation.

The first theory would be difficult to prove or disprove conclusively; on the other hand some evidence does exist in favor of the second theory. Single spore or colony isolations from a given parent culture, when tested for penicillin activity, show a marked diversity in this particular property. The tendency is toward a reduction, but isolates roughly equal to the parent culture are also found. Numbers of substrains having no, or very little, penicillin activity can be readily isolated. Occasionally substrains appreciably better than the parent will be found, but this occurs in a very small percentage of the total numbers tested.

An example of the type of cultural and physiological variation obtained in substrains is given in the following summary of three experiments. Different

types of colonies as described were isolated by plating out three stock strains of *P. notatum* which were compared in duplicate for penicillin-producing ability in brown sugar medium (see Protocol A).

PROTOCOL A

Exp't. I. Good penicillin-producing parent strain #7

SUBSTRAIN NO.	APPEARANCE ON SABOURAUD'S AGAR	PENICILLIN AFTER 11 DAYS
		Florey units/ml.
1	Abundant dark green sporulation	20
2	Dark grey, few spores	5
3	White, few spores	16
Parent	White, few spores	16

Exp't. II. Poor penicillin-producing parent strain #5

SUBSTRAIN NO.	APPEARANCE ON SABOURAUD'S AGAR	PENICILLIN* AFTER	
		4 days	6 days
1	Poorly sporulated, yellow drops on surface	>167	250
2	Poorly sporulated, yellow drops on surface	>167	250
3	Poorly sporulated, yellow drops on surface	167	250
4	Poorly sporulated, yellow drops on surface	>167	250
5	Poorly sporulated, yellow drops on surface	>167	250
6	Poorly sporulated, yellow drops on surface	>167	250
7	Abundant blue spores, no yellow drops	50	125
8	Abundant green spores, no yellow drops	<50	<10
9	Abundant green spores, no yellow drops	<50	<10
10	Abundant green spores, no yellow drops	<50	<10
11	Abundant green spores, no yellow drops	<50	<20

Exp't. III. Poor penicillin-producing parent strain #8

1	Abundant blue spores, no yellow drops	50	100
2	Abundant blue spores, no yellow drops	50	125
3	Abundant blue spores, no yellow drops	50	100
4	Abundant blue spores, no yellow drops	50	100
5	Abundant blue spores, no yellow drops	50	125

* Expressed as reciprocal of dilution of culture filtrates causing inhibition of *Staphylococcus aureus* H in nutrient broth in 20 hrs. at 37°C. For purposes of rough comparison, inhibition at a dilution of 1-100 is equivalent to 1 Florey unit.

Thus, the different parent cultures consisted of mixtures of substrains of varying penicillin-producing capacities. In experiment I two out of the three different colony type isolates produced penicillin at least as well as the parent strain; the third was definitely inferior. In Experiment II three types of colonies totaling 11 separate cultures were isolated and tested. The various isolates of any one type all behaved alike in penicillin-producing power. Those cultures with characteristic abundant green sporulation were unable to produce

culture may be used indefinitely, uniformity of culture potency is insured for a long time. In practise, different tubes of the soil cultures are used daily, i.e., on a stagger system, so that if something should go wrong with a given tube, only the liquid cultures for that day would be bad while those on subsequent days, originating from different tubes, would be good.

As mentioned earlier, it is important to stop incubation of spore cultures within one day after maximum sporulation occurs, which generally takes 3 to 4 days; otherwise the secondary vegetative development begins. Spores so prepared can be preserved with full penicillin activity for many months by storing in a refrigerator. The bright green spore color tends to change into a grey or brownish green with age, but this is without effect on the activity of the spores.

Production of penicillin in synthetic media

Two-liter Erlenmeyer flasks containing the modified Czapek-Dox medium previously described for penicillin production (Abraham, *et al.*, 1941) were inoculated with *P. notatum*, incubated at 25°C. and tested by ordinary dilution methods for antibacterial activity against *Staphylococcus aureus* in nutrient broth. From an initial value of approximately 6.5 the pH of the medium fell slowly to 3.0–4.0 but, contrary to the description of the Oxford investigators, failed to rise up to pH 7.0–8.0 and remained at the low pH throughout. Neutralized samples of the cultures showed high antibacterial activity and batches of such cultures ranging from 10 to 100 liters were then extracted¹ with amyl acetate at pH 2 according to the Oxford procedure for the recovery of penicillin. The antibacterial activity could not, however, be extracted by this procedure, and it was postulated that penicillin in these cultures existed in a non-extractable form, presumably bound with a high molecular substance. The mold growths appeared quite different from those of Abraham, *et al.* (1941). The pellicles were thin, without wrinkles or spores, and the liquid was practically colorless, only a faint yellow tinge appearing. When the basal modified Czapek-Dox medium was supplemented with yeast extract, brain-heart infusion, etc., the behavior of the cultures resembled that of the Oxford workers, and the antibacterial activity of the liquid was extractable at pH 2 with organic solvents and in general showed the characteristic properties of penicillin.

Experimentation revealed that the formation of non-extractable antibacterial activity was always associated with a low pH of the culture medium and one which remained low (pH 3–4). A number of different tests in the basal Czapek-Dox medium in which tap water was used, different batches of chemicals, heavy metals, etc., showed that the failure of the pH to rise was due to the excessive purity of the ingredients of the medium. When impure or crude chemicals are used in the medium, the pH always rises normally. These impurities are primarily traces of heavy metals, particularly zinc and also iron, copper and manganese. Introduction of these elements into the basal medium evokes the typical culture changes reported by the Oxford workers. Organic supplements

¹ We wish to thank Dr. R. L. Peck for these extraction experiments.

accomplish the same change. The surface pad was much heavier and more wrinkled, and sporulation and yellow pigmentation of the medium were present. The pH rose to 8.0–8.5. Antibacterial activity corresponding to authentic penicillin could be extracted from such cultures.

The isolation of notatin by Coulthard *et al.* (1942) provides an explanation for the above discrepancies. Notatin [also called penatin (Kocholaty, 1942), penicillin B (Roberts, *et al.*, 1943) and coli factor (Waksman and Woodruff, 1942)] is an antibacterial substance distinct from penicillin but also found in *P. notatum* cultures. It is active against gram-negative bacteria; penicillin is active primarily against gram-positive organisms. Its properties leave no doubt that the unextractable antibacterial activity described above was identical with notatin. Thus, certain strains of *P. notatum* may produce two separate antibacterial substances, and the cultural conditions govern which is formed. Under acid conditions notatin is formed, and in neutral or alkaline media true penicillin is formed. It is probable that different strains will vary in their relative ability to produce either one.

Cultures in synthetic media may produce notatin only during the early stages, and although penicillin is formed as the culture ages, the notatin does not increase significantly but may persist, so that in the later stages both antibacterial substances are present (analysis of data published by Kocholaty, 1942). In our experiments the early formation of notatin in synthetic medium is correlated with a fall in pH to about 3–4. However, under conditions where the pH value does not fall to this level and remain there for a few days but instead falls only to 4.2–4.6 and remains at that level for a much shorter time before rising rapidly to pH 7–8, notatin is not formed, and the whole of the antibacterial activity of the culture filtrates is due to penicillin.

Notatin is a flavoprotein which oxidizes glucose to gluconic acid, and, indeed, for its antibacterial effect requires glucose (or other carbohydrates) (Coulthard, *et al.*, 1942; Roberts, *et al.*, 1943). Although the assay of mold filtrates containing notatin is made in a carbohydrate-free medium (nutrient broth), enough carbohydrate is carried over in the sample to produce the effect.

Of all the synthetic or nearly synthetic media tested, the brown sugar medium of Hobby, Meyer and Chaffee (1942) was decidedly superior for penicillin activity. Its composition is as follows: dark brown sugar, 2 per cent; NaNO_3 , 0.35; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; KCl , 0.05; KH_2PO_4 , 0.15; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.015. It was found later that an increase to 0.60 per cent NaNO_3 was an improvement for penicillin activity. Growth is considerably more rapid and abundant in brown sugar and is accompanied by intense yellow pigmentation (chrysogenin). Good penicillin activity is rarely found in the absence of this pigmentation. Undoubtedly the impurities in brown sugar are of the right kind, amount or balance for penicillin formation. Different grades of brown sugar are progressively better for penicillin activity according to the degree of impurity. Figure 1 shows the course of change of penicillin formation, pH, dry cell weight and residual sugar during a typical brown sugar experiment in 2-liter Erlenmeyer flasks containing 500 ml. medium. The acidity falls in the early stages from

pH 5.5 to 4.5 and then rises rather rapidly up to approximately 8.0 and frequently to 8.5–8.8. The acidity is due to the formation of gluconic acid; and its consumption later, together with nitrate ion, leaves an accumulation of sodium ion, which raises the pH. Penicillin accumulation began on the fifth day and rose rapidly to its peak on the eleventh to the thirteenth day followed by a rather rapid loss in activity thereafter. Growth paralleled sugar consumption, and maximum penicillin formation appeared to be reached in this experiment only after maximum growth was attained. Practically all the sugar was consumed before maximum penicillin accumulation occurred. The reducing power of these filtrates never entirely disappears, some small amount of non-fermentable reducing substance apparently being formed as a result of growth of the mold.

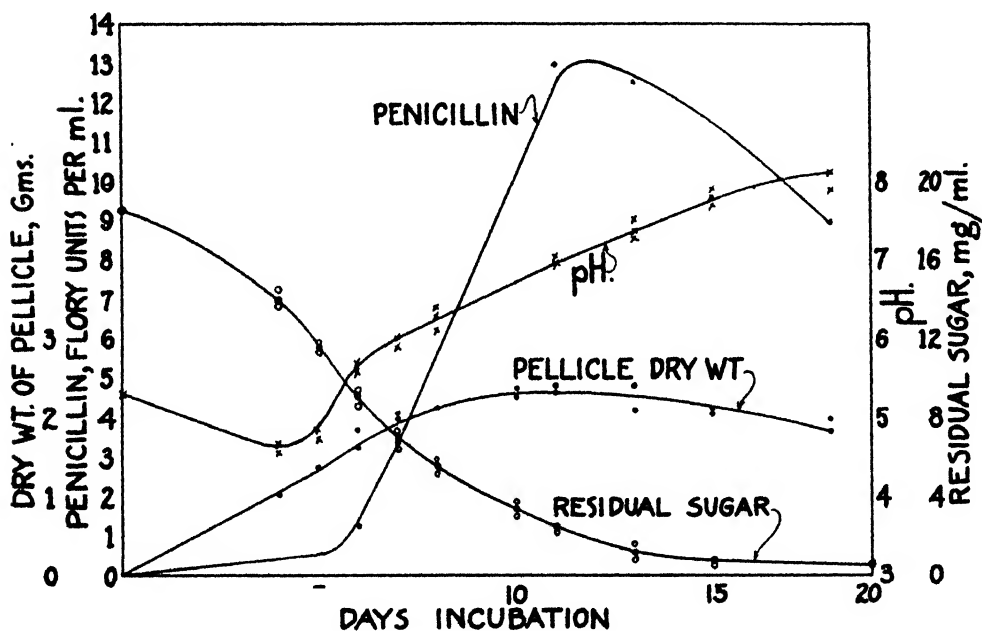


FIG. 1

In this experiment it amounted to 5 per cent of the sugar consumed. Periodic analyses showed that assimilation of nitrate, phosphorus and magnesium paralleled growth, and in this experiment after 13 days the following amounts of these minerals remained unconsumed (expressed in per cent of that initially present): NaNO_3 , 59; KH_2PO_4 , 81; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 91.

A large number of modifications in the basal medium failed to achieve any noteworthy improvement in the brown sugar medium. It was found, however, that the FeSO_4 and KCl may be eliminated entirely and the KH_2PO_4 content lowered considerably without reducing penicillin activity of the broth.

Higher levels of brown sugar failed to increase activity and, in fact, lengthened considerably the time required for attainment of maximum activity. This is due to the fact that higher carbohydrate concentration causes the accumulation

of larger amounts of gluconic acid so that the pH rise occurs slowly and may not occur at all. *P. notatum* preferentially oxidizes glucose to gluconic acid and so long as enough glucose is available it does not further oxidize the initially-formed gluconic acid. Since the production of penicillin activity always takes place only between approximately pH 6.0 and 8.5, this accumulation of acid is unfavorable for rapid formation of penicillin.

In our experience 0.6 per cent NaNO_3 is optimum even though as little as 0.15 per cent is incompletely assimilated for growth by the mold. The higher concentrations (0.45 to 0.60 per cent) of NaNO_3 invariably induce the pH to rise more rapidly. Lower concentrations progressively lead to greater acidity and prolong time for the pH rise. In a typical experiment, after 7 days 0.15, 0.30, and 0.60 per cent NaNO_3 showed pH 4.0, 7.8 and 8.2, respectively. It appears that a luxury consumption of nitrate ion occurs, leaving an excess of sodium ion in solution and accelerating the overall metabolism of the mold. The presence

TABLE 1
*KH₂PO₄ concentration and penicillin formation**
Brown sugar medium

PER CENT KH_2PO_4	11 DAYS		13 DAYS		PELLETCLE WEIGHT	PHOSPHORUS ADDED AS KH_2PO_4	PHOSPHORUS ASSIMILATED
	Florey units per ml.	pH	Florey units per ml.	pH			
0.0	<0.7	4.3	<0.1	4.1	gms. nil	mg.	mg.
0.005	0.2	5.4	2.8	8.0			
0.01	6.7	8.1	9.8	8.3	3.55	23	20
0.03	17.3	8.5	16.7	8.9	3.39	68	58
0.05	16.6	8.5	16.3	8.8	3.21	114	78
0.15	14.8	8.5	17.0	8.8	3.28	342	104

* Results based on 500 ml. of brown sugar medium in 2-liter flasks.

of excess nitrate favors the further oxidation and conversion into cell material of initially-formed gluconic acid. It may be that trace impurities in the NaNO_3 are in part responsible for this favorable effect.

Table 1 shows the effect of phosphate concentration on penicillin formation in brown sugar medium. While 0.01 per cent was not limiting for synthesis of cell material, it did limit penicillin formation. Although that amount of phosphate gave maximum growth, much more phosphate was assimilated when it was available (0.03 per cent), and this led to increased penicillin accumulation. Thus, the favorable effect of phosphorus on penicillin formation appears due not solely to synthesis of more cell material but also to effects on the metabolism of the mold.

The superiority of brown sugar over pure sucrose is not entirely due to the mineral impurities of the former. Brown sugar was ashed over a flame and then in a muffle furnace at 500°C. The ash was dissolved in a minimum of HCl and an aliquot of this neutralized solution equivalent to 2 per cent brown sugar

added to a 2 per cent C.P. sucrose-mineral medium. A control was also run with neutralized acid alone. The ash, when added to pure sucrose, could not replace whole brown sugar, thus indicating the presence of an organic fraction in brown sugar which promotes penicillin formation.

Glycerol was tried as a source of carbon for increased cell material, because it is readily used for growth by *P. notatum* and would not lead to gluconic acid formation. Consumption of nitrate during its utilization should also favor the rise in pH. In one experiment at 13 days in 2-per-cent brown-sugar medium containing various concentrations of glycerol, the activity in 2.5 per cent glycerol was 20 Florey units per ml. as against 9 in controls without glycerol. Pellicle weight increased almost proportionally to the concentration of glycerol, about twice as much growth being made at that concentration which gave twice as much penicillin as controls. There was a fairly narrow peak of maximum penicillin formation; glycerol concentrations over 2.5 per cent caused activities to drop off sharply despite the abundance of growth. Final pH values were fairly constant (7.5-7.7) over the range of maximum penicillin formation. A different

TABLE 2
Effect of grade of glycerol on penicillin formation in brown sugar medium

GRADE OF GLYCEROL*	FLOREY UNITS PER ML.					
	9 days	11 days	13 days	14 days	17 days	20 days
"High test"	8	11	25	22.5	13.5	10
U.S.P.	<2	<6	6	5	>10	7.5

* 1.5 per cent added to 2 per cent brown sugar medium.

lot of glycerol, however, did not yield the same beneficial effect on penicillin formation (table 2).

Other irregularities in penicillin formation were noted in comparing distilled and tap water. For a period of several weeks in brown sugar medium, alone or supplemented with glycerol, and even in organic media, tap water repeatedly proved definitely superior to distilled water. Then, gradually the effectiveness of the tap water became lowered to the point where it was no better than distilled water. This particular sequence of events occurred during the winter and spring months in 1942, the change being rather strikingly correlated with the advent of warm weather. Presumably, the composition of the tap water became considerably modified as a result of the seasonal change in soil conditions.

Zinc and penicillin formation

These findings, together with the differences between different lots of glycerol, pointed to impurities in glycerol and tap water as the agents responsible for the irregularities noted above. Traces of heavy metals are known (Foster, 1939) to exert profound influences on the growth and metabolism of fungi. Among the most important are zinc, iron, copper and manganese. Zinc proved to be of great importance in growth and penicillin formation by the strain of *P. notatum* used in this work. The following experiments typify the zinc effect.

Two-per-cent brown-sugar medium containing 2 per cent reagent glycerol was prepared with reagent chemicals and distilled water and 85 ml. apportioned in 250 ml. flasks. The various heavy metals were added as in table 3. The data show that zinc had a marked effect on penicillin formation both in the absence and presence of the other heavy metals, whereas manganese, iron and copper were without significant effect singly or combined. There was a definite concentration effect, penicillin production increasing up to 1 to 3 mg. $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ per liter and falling off markedly at higher concentrations. This concentration of added zinc remained optimum, irrespective of the presence of different amounts of the other heavy metals in various combinations. Growth was also markedly

TABLE 3
Heavy metals and penicillin formation

HEAVY METAL SUPPLEMENTS (MG./L.)				8 DAY ASSAY	
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	$\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$	$\text{FeSO}_4 \cdot 6\text{H}_2\text{O}$	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	Florey units per ml.	pH
0	1.0	1.0	0.5	8.0	7.0
0.1	1.0	1.0	0.5	9.0	7.3
0.3	1.0	1.0	0.5	15.3	8.2
1.0	1.0	1.0	0.5	21.9	8.4
3.0	1.0	1.0	0.5	21.3	8.8
10.0	1.0	1.0	0.5	10.8	8.8
30.0	1.0	1.0	0.5	<3.8	8.9
100.0	1.0	1.0	0.5	<3.8	8.8
0	0	0	0	9.0	7.1
1.0	0	0	0	18.6	8.8
10.0	0	0	0	13.8	8.8
0	10.0	10.0	5.0	9.0	7.3
1.0	10.0	10.0	5.0	19.2	8.4
10.0	10.0	10.0	5.0	9.0	8.8
0	1.0	10.0	5.0	7.5	7.5
1.0	1.0	10.0	5.0	14.5	8.5
10.0	1.0	10.0	5.0	12.9	8.8
Tap water control.....				11.1	8.4

increased up to the 10 mg. $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ level, higher concentrations already becoming toxic. The basal medium probably contained minute amounts of zinc before the supplements were added since some meager growth was made in the controls. It was slower and much less abundant than when zinc was added, but even this amount of growth was made possible only because of the minute amounts of zinc present as impurities in the medium. (Growth of *P. notatum* can be eliminated almost entirely when the medium is carefully purified to eliminate zinc.) Presumably the brown sugar contained amounts of zinc undetectable by spectrographic examination (below 1 p.p.m.) since some slight growth takes place in the basal medium, which would not be so in the complete absence of zinc. Thus, 1 to 3 mg. per liter of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.23–0.7 p.p.m. Zn

on) is close to the absolute optimum concentration of zinc for penicillin formation by this particular strain of *P. notatum* under the described conditions. Under certain circumstances this amount of zinc or portions of it may already be present in the medium as impurities in the water, chemicals, etc., and none or only smaller amounts may have to be added.

A medium deficient in zinc gives a thin, smooth, rubbery surface pellicle usually with very little sporulation so that the pellicle is white. The pH usually falls to about 3.5–4.0 and rises very slowly thereafter, if at all. With amounts of zinc approaching optimum, growth is considerably accelerated, the surface pellicle is much thicker and wrinkled and abundant green sporulation occurs. A characteristic invariably associated with the presence of sufficient zinc is the formation of the yellow pigment, chrysogenin, which imparts a deep yellow color to the medium. Rapid and abundant formation of penicillin never occurs in surface cultures without formation of the marked yellow pigment, although the reverse is not necessarily true. Zinc-deficient cultures show little yellow pig-

TABLE 4
*Zinc and penicillin formation with time**

ZnSO ₄ · 7H ₂ O mg./l.	FLOREY UNITS PER ML.			
	6 days	8 days	11 days	18 days
0	2.1	7.1	15.3	14.4
0.3	7.2	14.1	19.6	22.8
1.0	12.0	14.8	19.6	20.4
3.0	13.3	10.0	15.3	16.5
10.0	3.5	3.8	4.6	3.2

* Tap water-brown sugar medium containing 1.5 per cent glycerol. 500 ml. in 2-liter flasks.

ment formation, and the amount formed is roughly proportional to the concentration of zinc even up to 25 p.p.m. zinc. At this high zinc level growth is considerably retarded, and the pellicle is very fragile and brittle. It is noteworthy (table 3) that in every case the pH of zinc-deficient cultures was considerably lower than that of cultures containing adequate zinc.

Penicillin activity in suboptimal zinc cultures upon prolonged incubation tends to approach finally that formed in zinc cultures in one-third to one-half the time (table 4). Thus, in effect, zinc simply accelerates the formation of penicillin, probably because it accelerates the rise in pH through oxidation of gluconic acid by the mold.

Our data bear out the idea that the rôle of zinc in penicillin production appears to be concerned with oxidative metabolism of the mold. The presence of zinc enables the mold to oxidize the carbohydrate to completion for utilization for energy and cell synthesis. Zinc appears to catalyze this reaction (Foster, 1939). In the absence of this element or in cultures deficient in it, glucose is oxidized to gluconic acid which accumulates and lowers the pH to 3.5 to 4.0. The acid is

then only slowly further oxidized, and the pH rise lags (table 3). Since the optimum pH range for penicillin production lies between 6 and 8, the effect of zinc in accelerating the rise in pH is critical.

A certain balance exists between the concentrations of carbohydrate and zinc. As mentioned above, higher carbohydrate concentrations cause the accumulation of more gluconic acid and consequently lower the pH. Within limits, the addition of more zinc tends to counteract this effect, and the duration of the low pH values in such cultures is appreciably shortened.

The depth of liquid in any vessel, especially the ratio between volume of medium and its surface area has an important influence in the final yield of penicillin. Table 5 compares results obtained with flasks of different sizes containing different depths of liquid. The shallower layers are most favorable for penicillin formation, but in the four largest size flasks where the volume/area ratio was constant the smaller flasks gave significantly better and more rapid

TABLE 5
Depth-area influence on penicillin formation
(Brown sugar medium)

FLASK SIZE	VOLUME OF MEDIUM	VOLUME AREA	FLOREY UNITS/ML.		pH	
			5 days	7 days	5 days	7 days
ml.	ml.					
125	40	6.2	21.2	38.4	7.4	8.2
125	66	12.9	11.6	20.0	7.4	7.6
250	85	8.9	17.8	29.6	7.4	7.8
250	105	12.7	9.8	22.8	7.4	7.8
500	175	12.8	10.8	19.6	7.2	7.5
1000	310	12.7	9.4	17.6	7.2	7.6
2000	500	12.8	7.6	14.8	7.0	7.6

penicillin formation. Size of vessels and volume of medium are important considerations in interpreting penicillin results.

Other carbon and nitrogen sources

A variety of other carbon sources were tested as supplements to brown sugar media and as the sole carbon sources themselves. These include: glucose, lactose, maltose, sodium citrate, succinate, acetate, pyruvate, oxalacetate, gluconate, lactate, malate, ethyl alcohol and mannitol. None of these treatments was superior to the plain brown sugar medium, and only the first three approached it.

Out of numerous pure sources of nitrogen, including the individual amino acids, nitrate nitrogen is superior for penicillin formation by *P. notatum* in synthetic media.

SUMMARY

Different strains of *Penicillium notatum* differ markedly in penicillin-producing ability, and selection of the most potent strains is of primary importance for

maximum penicillin production. *Active strains tend to degenerate or lose their capacity to produce penicillin, especially after continued serial transfer on laboratory media. This degeneration can be eliminated by reducing vegetative transfers, and a practical system for doing this is described. Plating procedures on active cultures yield isolates with different degrees of penicillin activity.

Under conditions where the acidity of the medium reaches pH 3-4 and remains there for some time before rising, notatin is formed by *P. notatum*. Penicillin is produced when the pH does not fall so low and rises rapidly to 6.0-8.5. Extreme purity of the medium ingredients induces the appearance of the low pH and formation of notatin. The presence of certain trace elements, notably zinc under these conditions, and also organic supplements, favors the rapid rise in pH and formation of penicillin. Zinc in particular acts in this manner by catalyzing the complete oxidation and utilization of glucose by the mold, thus preventing the accumulation of gluconic acid which is responsible for the fall in pH of the medium.

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NICOTINIC ACID REQUIREMENTS OF CERTAIN YEASTS

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Knowledge of the accessory growth factors concerned in the physiology of lactose-fermenting yeasts was needed in an investigation of fermentation that was being conducted by the Bureau of Dairy Industry. Since information on nicotinic acid requirements for the growth of yeasts not fermenting lactose was at best fragmentary and incomplete and information on lactose-fermenting yeasts was completely lacking, a study was undertaken to develop the pertinent knowledge.

It has been known since 1913 that cells of the ill-defined species, *Saccharomyces cerevisiae*, contain nicotinic acid. Funk (1913) obtained nicotinic acid as a constituent of his "vitamine" fraction from yeast. With this knowledge of the nicotinic acid content of yeast, it would seem that investigators would have become interested in the growth response of living cells. However, a careful search of the literature has revealed very few publications on the growth response of yeast to nicotinic acid. Schultz, Atkin and Frey (1938) stated that nicotinic acid has no "bios" effect on strains of *S. cerevisiae*. Fink and Just (1939) showed that *Torula utilis*, an organism which does not ferment lactose, can synthesize the pyridine ring of nicotinic acid from such simple sources as ethanol, acetic acid, fermentable carbohydrates, ammonia nitrogen, and nutritive salts. Kögl and Borg (1941) found that the growth of the Königsgist or Strain M yeast is not stimulated by nicotinamide alone. Nicotinamide plus thiamine produced a slight stimulation which probably was caused by the thiamine rather than the nicotinamide. Leonian and Lilly (1942) cultivated yeasts which had been trained to grow on a completely synthetic medium devoid of vitamins. As will be shown later in this paper, it is unnecessary to "train" yeasts not fermenting lactose to grow in a medium free of nicotinic acid only.

Koser, Wright, and Dorfman (1942) mentioned, *en passant*, that nicotinamide was one of the factors necessary for rapid growth of one strain of *Torula cremoris*. No other information was found in the literature concerning the growth response of lactose-fermenting yeasts to nicotinic acid.

EXPERIMENTAL

Cultures. A total of 114 strains of lactose-fermenting yeasts was employed in this study. These strains have already been listed by Rogosa (1943). The taxonomic status ascribed to the cultures by the workers who isolated and studied them was provisionally accepted. In addition, the following seven strains of *Saccharomyces cerevisiae*, which do not ferment lactose, were studied: *S. cerevisiae* Hansen American Type Culture Collection #764, *S. cerevisiae*

Hansen A.T.C.C. #765, *S. cerevisiae* Hansen A.T.C.C. #4110, *S. cerevisiae* (F.B.) A.T.C.C. #7754, *S. cerevisiae* (O.P.) A.T.C.C. #7753, *S. cerevisiae* (G.M.) A.T.C.C. #7752, and Yeast "Steinberg" A.T.C.C. #4938.

Medium. The following medium was used in all experiments:

Casein hydrolysate (dry weight).....	5 g
Sodium acetate.....	6 g
Glucose.....	20 g
l-Asparagine.....	250 mg
l-Tryptophane.....	50 mg
l-Cystine.....	100 mg
Guanine hydrochloride.....	5 mg
Adenine sulfate.....	5 mg
Xanthine.....	5 mg
Uracil.....	5 mg
Salt solutions A and B; each.....	5 ml
Thiamine hydrochloride.....	200 micrograms
Riboflavin.....	200 micrograms
Ca pantothenate (d).....	200 micrograms
Pyridoxine hydrochloride.....	200 micrograms
p-amino-benzoic-acid.....	200 micrograms
Inositol.....	1000 micrograms
Choline chloride.....	1000 micrograms
Nicotinic acid (when supplied).....	1000 micrograms
Folic acid ¹ (100 per cent calculated potency).....	0.5 microgram
Biotin or biotin methyl ester.....	0.5 microgram
Lactic acid (0.6 ml.) to yield a pH of 4.8-5.0	
Distilled H ₂ O to yield a total volume of 1000 ml.	
Salt solution A: K ₂ HPO ₄ , 25 g; KH ₂ PO ₄ , 25 g; KI, 2.5 mg; H ₃ BO ₃ , 25 mg; H ₂ O to 250 ml.	
Salt solution B: MgSO ₄ · 7H ₂ O, 10 g; NaCl, 0.5 g; FeSO ₄ · 7H ₂ O, 0.5 g; Mn SO ₄ · 4H ₂ O, 0.5 g; CuSO ₄ · 5H ₂ O, 2.5 mg; ZnSO ₄ , 25 mg; H ₂ O to 250 ml.	

Preparation of medium. Labco "Vitamin-Free" casein was refluxed for 3 hours 3 successive times with 95 percent ethanol. An alternative procedure was to re-dissolve and re-precipitate the casein a minimum of 4 times. The alcohol treatment proved to be just as efficient and more convenient to use. The casein was dried and 100 g. was hydrolyzed with 500 ml. of 25 per cent H₂SO₄ by heating in the autoclave for 10 hours at 15 lbs. pressure. After cooling, the hydrolysate was stirred for 20 minutes with Norit-A (10 mg./g.) at a pH of approximately 1.0. This adsorption was repeated 3 times. Excess H₂SO₄ was removed by means of Ba(OH)₂ · 8H₂O until a pH of 5.2 was attained. The hydrolysate was diluted to a volume of 1 liter. C.P. glucose was used and in some experiments C.P. glucose in solution was treated with Norit-A and recrystallized twice. C.P. l-asparagine was dissolved to a concentration of 10 mg./ml. l-Cystine (2 mg./ml.) was dissolved in distilled H₂O without heating by adding enough HCl to effect solution. Guanine hydrochloride, adenine sulfate, xanthine, and uracil were dissolved in a concentration of 1 mg./ml. by prolonged heating in distilled H₂O acidified with enough HCl to effect solution.

¹ Supplied through the courtesy of Dr. R. J. Williams of the University of Texas.

Thiamine hydrochloride was dissolved in acetate buffer (pH 4.5); the other vitamins in distilled H₂O. All solutions were preserved under toluene in the dark at 10°C. In all experiments in which the growth response of an organism was to be tested to increasing concentrations of nicotinic acid, the nicotinic acid solutions were made on the day of the experiment.

Every lot of medium was made personally by the writer subject to a system of rigid controls for each reagent. The medium was tubed in 8 ml. quantities in ordinary bacteriological test tubes, which were plugged with cotton, and sterilized for 15 minutes at 15 lbs. steam pressure.

Glassware. All glassware employed was scrupulously clean and in final critical experiments was washed by the usual laboratory facilities and subsequently rinsed at least 10 times in tap H₂O and 3 times in distilled H₂O.

Inoculum and incubation. The cultures used for inoculum were between 48 and 72 hours old at the time of inoculation. Both serial transfer and washed cell techniques were employed. Serial transfer was carried out by loop through at least four passages in the basal medium with and without the presence of nicotinic acid. In washed cell techniques the cells were centrifuged and washed 3 times in phosphate buffer at pH 6.5. The cells were then resuspended in phosphate buffer equivalent to 10 times the original volume of the culture. This cell suspension was shaken for a minute or two in a mechanical shaker. One drop of this suspension was used for inoculum and inoculations were carried out quickly in order to avoid settling of cells at the bottom of the pipette. All cultures were incubated without agitation at 30°C. in the dark.

Measurement of growth responses. Measurements of growth responses were made photometrically by means of a photoelectric photometer (Type F, American Instrument Company) with filter 51. When the tubes were ready for photometric measurements, they were removed from the incubator and placed at 0°C. for at least 20 minutes to stop growth. A series of tubes was corked, shaken, the bubbles of gas allowed to dissipate (approximately 1 minute), the contents of the tubes were poured into matched tubes, and the photometric measurements determined as percentage of light transmitted through the tube. These readings were converted to percentage of light absorbed by subtracting them from 100.

Repetition of experiments. Each experiment was performed at least 3 times and some experiments were repeated as many as 8 times.

RESULTS

Characteristic growth responses to nicotinic acid of the washed cells of a yeast not fermenting lactose, *Saccharomyces cerevisiae* (F.B.), and a lactose-fermenting yeast, *Mycotorula lactis* #130, are shown in figure 1.

Seven strains of *S. cerevisiae* were transferred by loop through 3 passages in a medium free from nicotinic acid. From the third passage, loop transfers were made into 2 sets of media, one containing adequate nicotinic acid and one free from nicotinic acid. Growth responses were determined after 16 hours of incubation. The results, which are summarized in table 1, show that it was un-

necessary to furnish nicotinic acid for the growth of these yeasts not fermenting lactose.

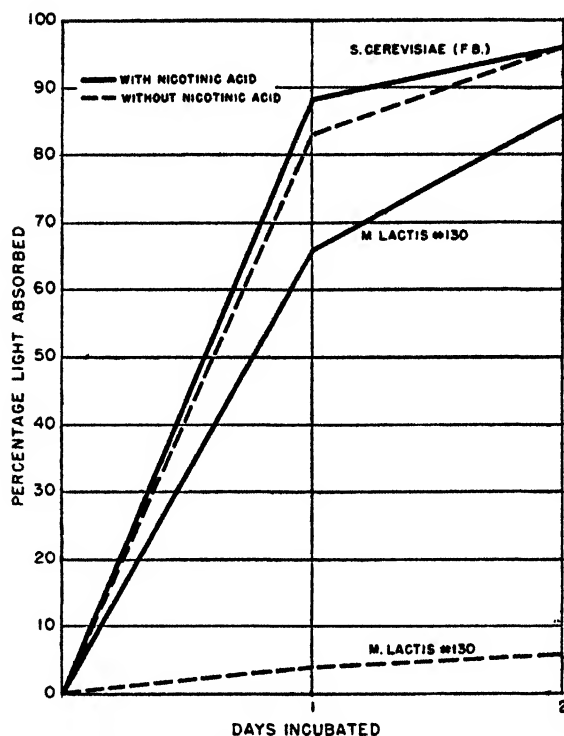


FIG. 1. GROWTH RESPONSES OF *SACCHAROMYCES CEREVISIAE* AND *MYCOTORULA LACTIS* IN THE PRESENCE AND ABSENCE OF NICOTINIC ACID

TABLE 1

Growth response of *Saccharomyces cerevisiae* in the fourth serial loop passage incubated for 16 hours in the presence and absence of nicotinic acid

CULTURE	PERCENTAGE LIGHT ABSORBED (UNINOCULATED CONTROL = 0)	
	Medium free from nicotinic acid	Complete medium
<i>Saccharomyces cerevisiae</i> Hansen #764.....	63	50
<i>Saccharomyces cerevisiae</i> Hansen #4110.....	68	66
<i>Saccharomyces cerevisiae</i> Hansen #765.....	53	61
<i>Saccharomyces cerevisiae</i> (old process) #7753.....	50	40
<i>Saccharomyces cerevisiae</i> (Gebrüder Mayer) #7752.....	44	45
<i>Saccharomyces cerevisiae</i> (F.B.) #7754.....	62	65
Yeast "Steinberg" #4938.....	49	46

One hundred fourteen strains of lactose-fermenting yeasts were transferred serially by loop through two parallel sets of media, one containing adequate nicotinic acid and one free from nicotinic acid. Incubation was for 7 days in each passage and transfers were made at the end of this time regardless of the

abundance or paucity of growth. Typical growth responses of 12 representative strains in the fifth serial loop passages are shown in table 2. These results show that an external source of nicotinic acid is indispensable for the continued growth of these lactose-fermenting yeasts.

TABLE 2

Growth response of lactose-fermenting yeasts in the fifth serial loop passage* incubated for 7 days in the presence and absence of nicotinic acid

CULTURE	PERCENTAGE LIGHT ABSORBED (UNINOCULATED CONTROL = 0)	
	Medium free from nicotinic acid	Complete medium
<i>Saccharomyces anamensis</i> #145.....	2	93
<i>Saccharomyces lactis</i> #131.....	2	90
<i>Saccharomyces fragilis</i> #15.....	2	86
Type F #93.....	6	93
<i>Monilia pseudotropicalis</i> (Castellani) #32.....	4	95
<i>Mycotorula lactis</i> #130.....	7	90
<i>Zygosaccharomyces lactis</i> #27.....	8	93
<i>Zygosaccharomyces lactis</i> #90.....	5	90
<i>Torulopsis kefir</i> #149.....	1	80
<i>Torula cremoris</i> #2.....	6	87
<i>Torula lactosa</i> #168.....	2	88
<i>Torula sphaerica</i> #13.....	7	95

* Seven days incubation between each transfer.

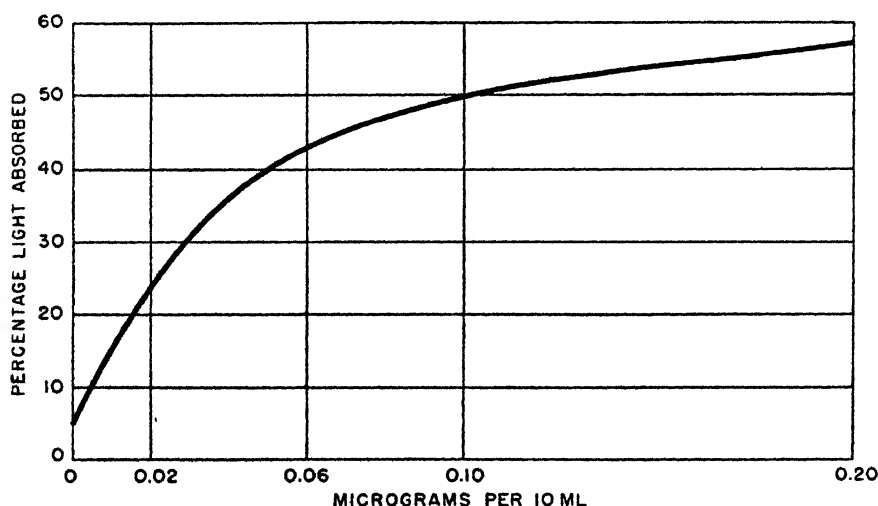


FIG. 2. GROWTH RESPONSE OF *MONILIA PSEUDOTROPICALIS* #32 TO INCREASING CONCENTRATIONS OF NICOTINIC ACID. INCUBATION 24 HOURS AT 30°C.

To obtain further evidence of the essentiality of nicotinic acid for the growth of lactose-fermenting yeasts, cells of *Monilia pseudotropicalis* (Castellani) #32 were washed 3 times and inoculated into a series of tubes containing graded concentrations of nicotinic acid. Inoculations were made into each concentration

in quadruplicate and tested after incubation for 24 hours. The result, which was characteristic of all lactose-fermenting strains tested, is shown graphically in figure 2.

DISCUSSION

Generally, growth of an organism may proceed for 1 or even 2 transfers in a deficient medium. This fact suggests the possibility of cell storage of vital materials that enable the cell to function until the vital materials are exhausted. For this reason serial transfer techniques were employed in addition to washed cell techniques. With some strains slight growth was noted on prolonged incubation in a medium free from nicotinic acid when washed cells were used for inoculum. On passing the cultures through serial transfer, there were only traces of growth at best. In general, however, the techniques yielded corroborative results.

The data show that lactose-fermenting yeasts cannot synthesize sufficient nicotinic acid to satisfy the normal needs of reproduction and indicate that yeasts which do not ferment lactose readily synthesize sufficient nicotinic acid for optimum growth.

The role of nicotinic acid or nicotinamide in coenzyme systems has been reviewed and emphasized by Schlenk (1942). In view of the differences in exogenous nicotinic acid requirements between lactose-fermenting and yeasts not fermenting lactose, it is probable that the vitamin-enzyme relations of the cell in fermentation are dissimilar for the two groups of organisms.

SUMMARY

Yeasts which do not ferment lactose do not require an exogenous source of nicotinic acid for growth. Lactose-fermenting yeasts, in contrast, require an exogenous source of nicotinic acid for growth.

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THE GROWTH REQUIREMENTS OF *LEUCONOSTOC MESAENTEROIDES* AND PRELIMINARY STUDIES ON ITS USE AS AN ASSAY AGENT FOR SEVERAL MEMBERS OF THE VITAMIN B COMPLEX.

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INTRODUCTION

Many investigators have found that the vitamin B complex is of major importance for the growth of lactic acid bacteria. Although the nutritional requirements of lactobacilli and streptococci have been given prominence, considerable work has also been done on the other members of this group of organisms. For example, *Leuconostoc mesenteroides* was shown to require pantothenic acid by Snell, Strong, and Peterson (1938), while Bohonos, Hutchings, and Peterson (1941, 1942) observed that added pyridoxin was stimulatory. Snell and Strong (1938, 1939) demonstrated that this organism produced relatively large amounts of riboflavin and that the addition of more of this vitamin was without beneficial effect.

The work described in this paper is an endeavor to contribute further to our knowledge of the importance of the vitamin B group in bacterial nutrition. The first section of the paper is concerned with studies on the vitamin requirements of *L. mesenteroides*, and the second, with attempts to utilize this bacterium for vitamin assays.

EXPERIMENTAL

A. Determination of vitamin requirements

I. Materials

A. Basal medium. The composition of the basal medium, given in table 1, was similar to that employed by Snell and Mitchell (1941).

Acid-hydrolyzed casein was prepared by adding 50 g. of vitamin-free casein¹ to 250 ml. of concentrated HCl and autoclaving the mixture for 10 hours at 15 lbs. pressure. After adjusting the pH to approximately 3.0 with NaOH, the suspension was stirred vigorously with 5 g. of norite for 30 minutes and then filtered.

Cystine hydrochloride was prepared by adding 3 ml. of concentrated HCl to 0.5 g. of cystine in order to effect solution of the latter and this was diluted with distilled water so that 1 ml. of the final solution contained 1 mg. of cystine.

B. Culture. Throughout the investigation *Leuconostoc mesenteroides* 535² was employed. Stock cultures were carried in a sucrose-peptone medium.

¹ The vitamin-free casein and vitamins were obtained from the S. M. A. Corporation.

² This strain was received from Dr. Carl S. Pederson, New York State Agricultural Experiment Station, Geneva, N. Y.

C. *Vitamin solutions.* Aqueous solutions of thiamin chloride, calcium pantothenate, pyridoxin hydrochloride, nicotinic acid, and crystalline biotin were prepared and stored in the refrigerator. Biotin was preserved under toluene in addition to being kept at the low temperature.

II. Methods

A. *General procedure.* Appropriate vitamin dilutions and double distilled water were added to a series of Evelyn photoelectric colorimeter tubes so that all contained equal volumes. Basal medium, prepared at twice the concentrations indicated in table 1, was added to each tube in a volume equivalent to that already present. Sterilization, effected by autoclaving at 15 lbs. for 15 minutes, was followed by cooling and inoculation; incubations were at 25°C. In this phase of the work the vitamins were used in concentrations of 0.1 μ g. each per ml. of medium.

TABLE 1
Basal medium for Leuconostoc mesenteroides

MATERIALS	GRAMS PER 100 ML.
Acid hydrolyzed casein (vitamin-free).....	0.5
Sodium acetate.....	0.6
Glucose.....	1.0
Cystine hydrochloride.....	0.01
Tryptophane.....	0.01
Inorganic salts:	
K ₂ HPO ₄	0.05
KH ₂ PO ₄	0.05
MgSO ₄ ·7H ₂ O.....	0.02
NaCl.....	0.001
FeSO ₄ ·7H ₂ O.....	0.001
MnSO ₄ ·7H ₂ O.....	0.001

The pH was adjusted to 6.8 in all cases.

B. *Inocula.* A 48-hour sucrose-peptone broth culture of *L. mesenteroides* was centrifuged, the supernatant was discarded, and the cells were resuspended in an equal volume of double distilled water. One ml. of this suspension was added to 99 ml. of water and 0.2 ml. of the latter served as the inoculum for each tube.

C. *Avidin inactivation.* Landy, Dicken, Bicking, and Mitchell (1942) described a method for using egg white (avidin) in ascertaining the biotin requirement of microorganisms. A modified procedure was used in the present investigation.

Egg white was dissolved in sterile physiological saline to give a final concentration of 30 per cent. Varying amounts of this solution were added aseptically to previously sterilized duplicate tubes containing basal medium and the four vitamins of the B group shown to be necessary. After inoculation and incubation for 48 hours, biotin was added to one of each pair of duplicate tubes and all were reincubated for another 36 hours.

D. *Growth determinations.* Turbidity as a measure of growth was determined in an Evelyn photoelectric colorimeter. The uninoculated medium control tube was set at a reading of 100 and all other tubes were compared to this standard. Lower colorimeter readings indicate greater degrees of turbidity and, thus, more growth.

III. Results

Six members of the vitamin B complex were examined individually and in combination for their effect on the growth of *L. mesenteroides*. Thiamin, riboflavin, biotin, nicotinic acid, calcium pantothenate, and pyridoxin were added to a series of tubes as indicated in the section on methods and incubated for a period of 72 hours. Good growth was obtained in the presence of all six vita-

TABLE 2
The effect of various combinations of vitamins on the growth of L. mesenteroides

TUBE	VITAMINS ADDED	COLORIMETER READINGS
1	Basal medium only—uninoculated	100
2	Basal medium only—inoculated	98
3	Bi, R, P, N, T, Py	70 ²
4	R, P, N, T, Py	71
5	Bi, R, P, N, T	87
6	Bi, R, P, N, Py	96 ³
7	Bi, R, P, T, Py	98 ¹
8	Bi, R, N, T, Py	96 ³
9	Bi, P, N, T, Py	69

R = riboflavin, Bi = biotin, Py = pyridoxin, N = nicotinic acid, T = thiamin, P = calcium pantothenate.

Readings taken 72 hours after inoculation.

mins; individually, none of these factors supported growth, indicating that more than one vitamin was required.

In order to demonstrate which, if any, of the six could be eliminated, an experiment was arranged as shown in table 2. A different vitamin was omitted from each of tubes 4 through 9, while tube 3 contained them all and tubes 1 and 2 contained none. Resultant good growth in tube 9 reveals that *L. mesenteroides* did not require added riboflavin, confirming the work of Snell and Strong (1938, 1939). The low reading obtained for tube 4 (no added biotin) seemed to show that this supplement was not an essential factor but subsequent investigation, described later in this paper, demonstrated that this was not the case. Tubes 6, 7, and 8 disclosed that thiamin, nicotinic acid, and calcium pantothenate, respectively, were necessary, since the lack of any one of these resulted in practically no growth. The turbidity obtained in the absence of added pyridoxin agrees with the results published by Bohonos and his coworkers (1941, 1942) who reported that *L. mesenteroides* synthesizes vitamin B₆ to a limited extent.

With regard to the biotin requirement, preliminary observations with con-

centrates had indicated that this vitamin was an essential factor for *L. mesenteroides*; however, the results of table 2 (tube 4) show that considerable growth occurred when it was not incorporated in the medium. Employing the procedure of avidin inactivation of biotin, it was found that this vitamin must be present before growth of the organisms would occur even though the other necessary factors were added. Table 3 illustrates this finding. After the primary incubation period of 48 hours, during which time the traces of biotin present in the basal medium were inactivated by combination with avidin, colorimeter readings revealed that practically no growth occurred in any tube. At the end of the second incubation period of 36 hours, complete reversal of the

TABLE 3

The biotin requirement of L. mesenteroides as shown by the egg white (avidin) inactivation method

TUBE	EGG WHITE ADDED	48-HOUR COLORIMETER READINGS	BIOTIN ADDED	84-HOUR COLORIMETER READINGS
	ml.		μg.	
1	0.60	98 ²	1.0	68 ²
2		98 ²	0.0	93
3	0.45	97 ²	1.0	74 ²
4		97 ¹	0.0	94
5	0.30	99	1.0	78
6		99	0.0	98
7	0.15	99	1.0	79 ¹
8		99 ²	0.0	99 ²

action of avidin was obtained where biotin had been added, while little or no change took place in those tubes which lacked this vitamin.

B. Establishment of standard assay curves

I. Materials

The materials employed in the experiments on the establishment of standard assay curves were prepared in the same manner as the corresponding materials utilized in section A, with two exceptions. First, a different sample of tryptophane was utilized in the preparation of the experimental basal medium; and second, for this work, stock cultures were carried in a medium of the same composition as that of the experimental basal medium, but which, in addition, contained nicotinic acid, calcium pantothenate, pyridoxin, and thiamin in a concentration of 0.0001 per cent each.

II. Methods

A. General procedure. Varying dilutions of the vitamin to be examined were added to a series of Evelyn photoelectric colorimeter tubes which contained 5

ml. of double strength basal medium. Adequate amounts of the other required vitamins were added to each tube and the contents of all tubes were diluted to 10 ml. with double distilled water. After sterilization at 15 lbs. for 15 minutes, the media were inoculated and incubated at 25°C.

B. *Inocula*. Inocula were prepared and used in the same way as previously described.

C. *Growth determinations*. Two methods were employed.

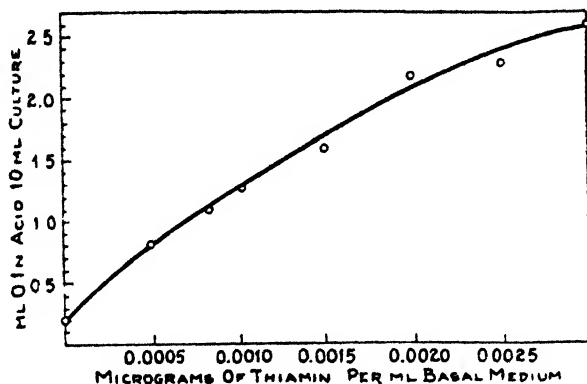


FIG. 1. STANDARD ASSAY CURVE FOR THIAMIN

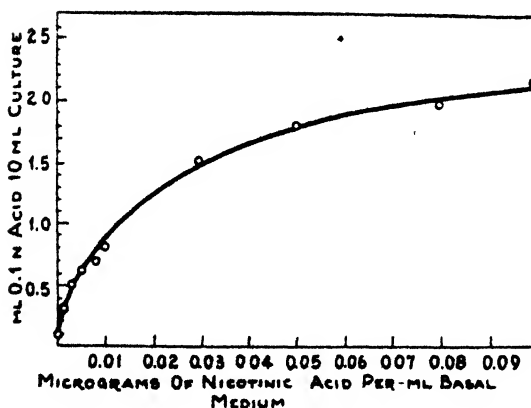


FIG. 2. STANDARD ASSAY CURVE FOR NICOTINIC ACID

1. Turbidity measurements were made with the Evelyn photoelectric colorimeter.

2. The acid produced during growth was determined by potentiometric titrations. In this method the contents of each of the tubes of the test were titrated with 0.1 N NaOH to the same pH as that of the uninoculated control.

III. Results

Having established the vitamin requirements of *L. mesenteroides*, the effect of incubation time on the amount of growth was ascertained. Prolonged incuba-

tion failed to produce significant changes in growth after 72 hours. It was decided, therefore, that the 72-hour incubation period would be used throughout the assay investigations.

Inasmuch as assays of various substances are dependent on the presence of adequate amounts of the other requirements, the problem of optimal vitamin concentrations was investigated. Results obtained disclosed that concentrations of 0.3 to 0.5 $\mu\text{g.}$ each of thiamin, nicotinic acid, calcium pantothenate, and

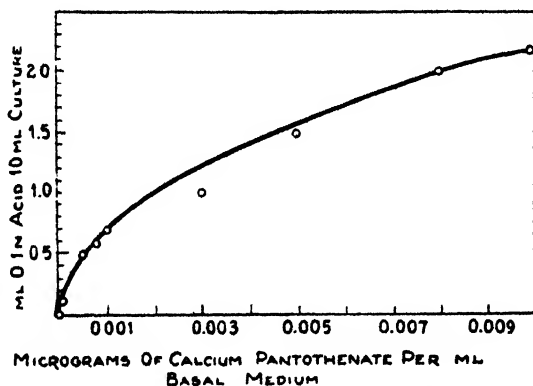


FIG. 3. STANDARD ASSAY CURVE FOR CALCIUM PANTOTHENATE

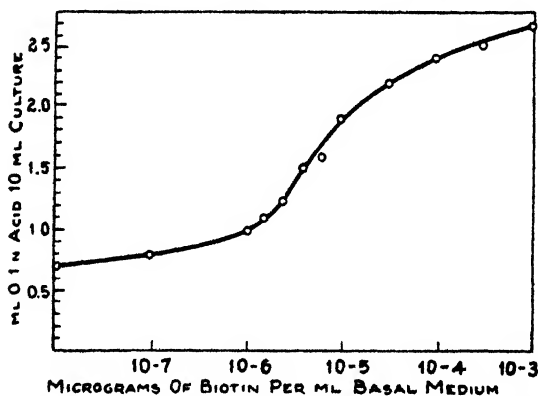


FIG. 4. STANDARD ASSAY CURVE FOR BIOTIN

pyridoxin per ml. of medium were satisfactory for excellent growth. One hundredth of a $\mu\text{g.}$ of biotin per ml. was utilized throughout, as previous work had demonstrated that amounts greater than this were without increased effect.

Typical standard assay curves for all five vitamins required by *L. mesenteroides* are presented in figures 1 through 5, in which titratable acidities are plotted against vitamin concentrations, while table 4 records these values and also includes turbidity determinations. In every case the vitamins, other than the one being examined, were each present in concentrations of 0.5 $\mu\text{g.}$ per ml. of

medium; biotin, however, was employed as indicated above. That acid production is indicative of growth of *L. mesenteroides* may be seen from the correla-

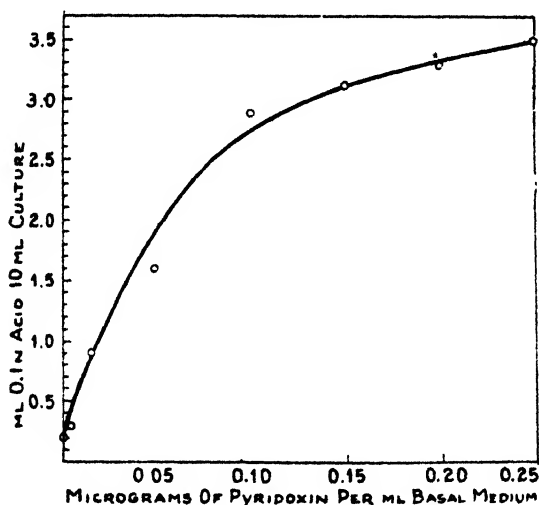


FIG. 5. STANDARD ASSAY CURVE FOR PYRIDOXIN

TABLE 4

The effect of the addition of various concentrations of several members of the vitamin B group on the growth and acid production of *L. mesenteroides*

TUBE	THIAMIN			NICOTINIC ACID			Ca PANTOTHENATE			BIOTIN			PYRIDOXIN		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
1*	0.0	100	0.0	0.0	100	0.0	0.0	100	0.0	0.0	100	0.0	0.0	100	0.0
2†	0.0	100	0.0	0.0	100	0.0	0.0	100	0.0	0.0	100	0.0	0.0	100	0.0
3	0.0	98	0.2	0.0	99 ^a	0.1	0.0	100	0.0	0.0	94	0.7	0.0	98	0.2
4	5×10 ⁻⁴	90	0.8	5×10 ⁻⁴	97 ^a	0.2	10 ⁻⁴	99 ^a	0.1	10 ⁻⁷	93	0.8	10 ⁻²	96 ^a	0.3
5	8×10 ⁻⁴	86	1.1	10 ⁻³	96 ^a	0.3	5×10 ⁻⁴	95	0.5	10 ⁻⁴	91 ^a	1.0	3×10 ⁻³	90	0.9
6	10 ⁻³	83	1.3	3×10 ⁻³	94 ^a	0.5	8×10 ⁻⁴	93 ^a	0.6	2×10 ⁻³	91	1.1	5×10 ⁻³	82	1.6
7	1.5×10 ⁻³	79 ^a	1.6	5×10 ⁻³	92	0.6	10 ⁻³	92	0.7	4×10 ⁻³	89 ^a	1.2	10 ⁻¹	70	2.9
8	2×10 ⁻³	75	2.2	8×10 ⁻³	90 ^a	0.7	3×10 ⁻³	87	1.0	6×10 ⁻³	86 ^a	1.5	1.5×10 ⁻¹	69	3.1
9	2.5×10 ⁻³	74	2.3	10 ⁻²	89 ^a	0.8	5×10 ⁻³	82 ^a	1.5	8×10 ⁻³	85 ^a	1.6	2×10 ⁻¹	67	3.3
10	3×10 ⁻³	71 ^a	2.6	3×10 ⁻²	82 ^a	1.5	8×10 ⁻³	80	2.0	10 ⁻²	83	1.9	2.5×10 ⁻¹	65	3.5
11	10 ⁻²	72 ^a	2.8	5×10 ⁻²	80 ^a	1.8	10 ⁻²	77 ^a	2.2	5×10 ⁻²	79	2.2	5×10 ⁻¹	66	3.5
12	5×10 ⁻¹	71 ^a	3.2	8×10 ⁻³	79 ^a	2.0	3×10 ⁻²	74 ^a	2.7	10 ⁻⁴	76	2.4			
13				10 ⁻¹	78 ^a	2.2	5×10 ⁻³	74	2.9	5×10 ⁻⁴	74	2.5			
14				2×10 ⁻¹	77 ^a	2.4	8×10 ⁻³	74 ^a	2.9	10 ⁻²	73 ^a	2.7			
15				5×10 ⁻¹	75 ^a	2.6	10 ⁻¹	74 ^a	2.8	5×10 ⁻³	72	2.9			
16							2×10 ⁻¹	74 ^a	2.8	10 ⁻¹	71	3.1			
17							5×10 ⁻¹	73 ^a	3.0						

* Basal medium only—uninoculated.

† Basal medium only—inoculated.

A, concentration of vitamin in $\mu\text{g.}$ per ml. of medium; B, colorimeter readings; C, ml. N/10 acid per tube.

tion existing between titratable acidities and colorimeter readings. While either method may be used for determining growth, the turbidity method has the advantage of requiring less time.

It should be mentioned that the use of a different sample of tryptophane in the assay work provided further confirmation for biotin requirement. With the newer tryptophane in the basal medium, little growth resulted in the absence of this vitamin and only when it was added did increased growth occur. Further, inoculated basal medium controls of the assay experiments failed to yield growth, in contrast to results obtained with the original sample of tryptophane.

DISCUSSION

From the data presented it is clear that good growth of *Leuconostoc mesenteroides* 535 was obtained when thiamin, nicotinic acid, calcium pantothenate, pyridoxin, and biotin were supplied in a suitable basal medium, and that omission of any one of these vitamins resulted in little or no growth. In the case of pyridoxin, however, considerable growth usually occurred even when it was not added (table 2). Occasionally, little turbidity was obtained in the absence of this vitamin as shown in table 4. Mention has already been made of the work of Bohonos and his coinvestigators (1941, 1942) concerning the B₆-synthesizing capacity of *L. mesenteroides*. These workers observed that pyridoxin is synthesized by this organism but at a rate insufficient to meet all the requirements of the cell. Added vitamin B₆, therefore, may be considered as a stimulatory rather than an essential factor.

Two reasons might be given for the growth obtained when biotin was not incorporated in the medium. First, this vitamin is exceedingly difficult to remove completely from the constituents of basal media and the presence of extremely minute amounts is sufficient to induce growth of bacteria which require it. Second, *L. mesenteroides* may synthesize biotin to a limited extent in a manner analogous to its production of pyridoxin. It has been found that a correlation exists between growth inhibition by avidin and an organism's biotin requirement. In general, those organisms which have been found to require biotin supplied in the culture medium are inhibited by avidin, while those which synthesize biotin are not affected. Table 3 demonstrates that the growth of *L. mesenteroides* was almost completely inhibited by avidin and that this inhibition was reversed by the addition of biotin. Evidence for the first explanation is afforded by these observations, since inhibition presumably would not have occurred had biotin been synthesized by *L. mesenteroides*. Further evidence for this point is given by the fact that little growth resulted in the absence of biotin when the newer sample of tryptophane was employed in the basal medium.

One of the noticeable features of the assay studies was the observation that *L. mesenteroides* failed to yield uniform responses to given levels of the vitamins. Individual experiments were repeated a number of times and gave the same relative but different absolute values. Data for each table and curve were therefore taken from the same experiment. Bohonos *et al.* (1942), reporting similar results, observed that *Lactobacillus casei* did not respond regularly to definite concentrations of pyridoxin. They considered the variations to be dependent upon the age of the culture, the number of subcultures from the stock

culture, the size of the inoculum, and the amount of vitamin B₆ present in the medium. *L. casei* was found capable of storing pyridoxin and, thus, larger inocula grew better than smaller inocula in the lower levels of added pyridoxin. Since *L. mesenteroides* synthesizes vitamin B₆, the size of inoculum accounted for the different absolute values to some extent.

Reference to the assay tables and the corresponding curves reveals that for all five vitamins colorimeter readings and titratable acidities were, within certain limits, nearly proportional to the concentration of the vitamin being determined. While the amount of each essential vitamin required for the stimulation of growth of *L. mesenteroides* varied from one vitamin to another, the quantity was very small in every case. This sensitivity to vitamin concentration makes it possible to dilute greatly an extract of material to be assayed, thereby removing or lessening the effect of growth-enhancing factors which might be present. In view of these facts, it is probable that *L. mesenteroides* can be satisfactorily utilized in assaying plant and animal tissues for the vitamins which it requires. Assays of this nature are under investigation.

SUMMARY

Using a medium in which all constituents except casein hydrolysate were chemically defined, it was found that thiamin, calcium pantothenate, and nicotinic acid were essential for the growth of *Leuconostoc mesenteroides* 535 and that pyridoxin exerted a stimulatory effect. Although growth resulted in the absence of added biotin, employment of the avidin inactivation technic demonstrated the biotin requirement of this organism.

With *L. mesenteroides* as the assay agent it was possible to establish standard assay curves for the vitamins essential to this organism. Turbidity readings and titratable acidities were well correlated and were, within certain ranges, nearly proportional to the concentration of the vitamin under test.

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A CYTOLOGICAL AND MICROCHEMICAL STUDY OF THIOBACILLUS THIOOXIDANS

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Thiobacillus thiooxidans is characterized by certain physiological peculiarities which have aroused wide interest since it was first isolated and studied by Waksman and Joffe (1922). This organism can oxidize elementary sulfur and thio-sulfates; it is able to grow at pH 1, and is inhibited by a pH ≥ 6 . Until recently, however, the morphology of the organism was restricted to such routine studies as measurement of cell dimensions, motility, and the gram reaction. Recent interest in its cell structure was stimulated by the need of a morphological basis to explain the mechanism by which the organism is able to attack elementary sulfur (Umbreit, Vogel and Vogler, 1942). According to those investigators, there is, at each end of the cell of *Thiobacillus thiooxidans*, a droplet of highly unsaturated fat which is responsible for the "dipolar" staining of the cell by certain staining procedures. "This fat globule is placed in contact with the sulfur particle, in such a manner that sulfur dissolves in it and is taken into the cell for oxidation." Later studies with the electron microscope (Umbreit and Anderson, 1942), however, did not bring out the "dipolar appearance" of the cell. The electron micrographs showed principally a mixture of cell forms varying from short, ellipsoidal cells ($1 \times 0.5\mu$) to elongated, cylindrical cells ($2-3 \times 0.5\mu$). The cells showed also considerable variation in transparency to the electron beam (presumably at an accelerating potential of 60 kv.), the majority being uniformly opaque to the electrons. Of particular interest are three cylindrical cells which showed an internal structure in the form of helicoidal bands, for similar bands were observed in a few of the large bacteria by Swellengrebel (1909) and by Dobell (1911), both of whom considered them as cell nuclei.

PRESENT INVESTIGATION

Culture and medium

The culture of *Thiobacillus thiooxidans* used in this investigation was received through the courtesy of Doctor Umbreit, of the University of Wisconsin, to whom we are also thankful for placing at our disposal his original electron micrographs, and the necessary information for culturing and handling the organism. The medium we used most frequently was that of Vogler and Umbreit (1941) in which $(\text{NH}_4)_2\text{SO}_4$ was substituted for NH_4Cl in a concentration containing about the same amount of nitrogen. The mineral solution was distributed in test tubes, in 10 ml. amounts, and sulfur, in the form of sterile flowers of sulfur, was added to each tube with a sterile loop. Cultures were initiated by inocula-

tion of each tube with 1 ml. of a 1-2 day old culture of the organism in a similar medium, and incubation at the temperature of the laboratory which varied between 25° and 30°C. We also used cultures grown at laboratory temperature on the thiosulfate agar medium of Waksman (1922).

Methods

The methods used in the present investigation are similar to those previously used by the author in studying the cell structure of other bacteria (Knaysi, 1941, 1942). The buffered dye solutions were prepared mostly by adding 1 ml. amounts of 1 per cent solutions of the dye in water to 4 ml. portions of buffer solutions prepared by mixing, in various proportions, 1 per cent solutions of monopotassium and dipotassium phosphate, or 0.1 *N* acid potassium phthalate and 0.1 *N* HCl. The final pH was determined with the glass electrode. Smears were prepared from liquid cultures, usually filtered through No. 4 Whatman paper to remove the sulfur particles, and observations were made mostly on wet preparations, unfixed, fixed by heat or by 95 per cent alcohol. Whenever necessary, the fixed smear was immersed for a few moments in a beaker of water to remove the acids transported from the medium. The microscopic and photomicrographic combinations and films are the same as those used previously (Knaysi, 1942).

Form, size and grouping of the cells

The *form* and *size* of the cell were studied in smears fixed by heat and stained by the method described (Knaysi, 1941) for the demonstration of the cell-wall. In the liquid, *sulfur medium*, young, actively growing cultures (fig. 1) consist of cells the majority of which are fairly uniform and have the shape of a short ellipsoid of revolution with the following characteristics (see Knaysi, 1941):

$$E = 1 - \frac{b^2}{a^2} = +0.44 \text{ to } +0.7$$

$$a = 0.6 \text{ to } 0.7\mu$$

$$b = 0.4 \text{ to } 0.5\mu$$

where *E* is the excentricity, *a* half of the major axis, and *b* half of the minor axis of the principal section. The volume ($V = 4/3 \pi ab^2$) of the cell varies, accordingly, from $0.34\mu^3$ to $0.6\mu^3$. Those cells probably correspond to "Type I" of Umbreit and Anderson (1942). As the cultures grow older, one observes a mixture of cellular forms including cylindrical or curved cells of different thickness, large spherical or ellipsoidal cells having the earmarks of chlamydo spores (microcysts), irregular cells, ghost cells, and cell debris. It may therefore be concluded that the cultures of *Thiobacillus thiooxidans* in the liquid, sulfur medium present similar sequence and mixture of cell forms to those observed in cultures of other bacteria. On the *thiosulfate agar medium*, the cells generally tend to be more elongated (fig. 2), with *a* varying from 0.8 to 1.2μ , *b* from 0.5 to 0.6μ , and *V* from 0.84 to $1.26\mu^3$.

The cells are mostly single or in pairs, often held in close proximity by the masses of slime in which they are imbedded. Smears made from thiosulfate agar slants often show a tendency, on the part of the cells, to lie parallel to one another.

Structure of the cell

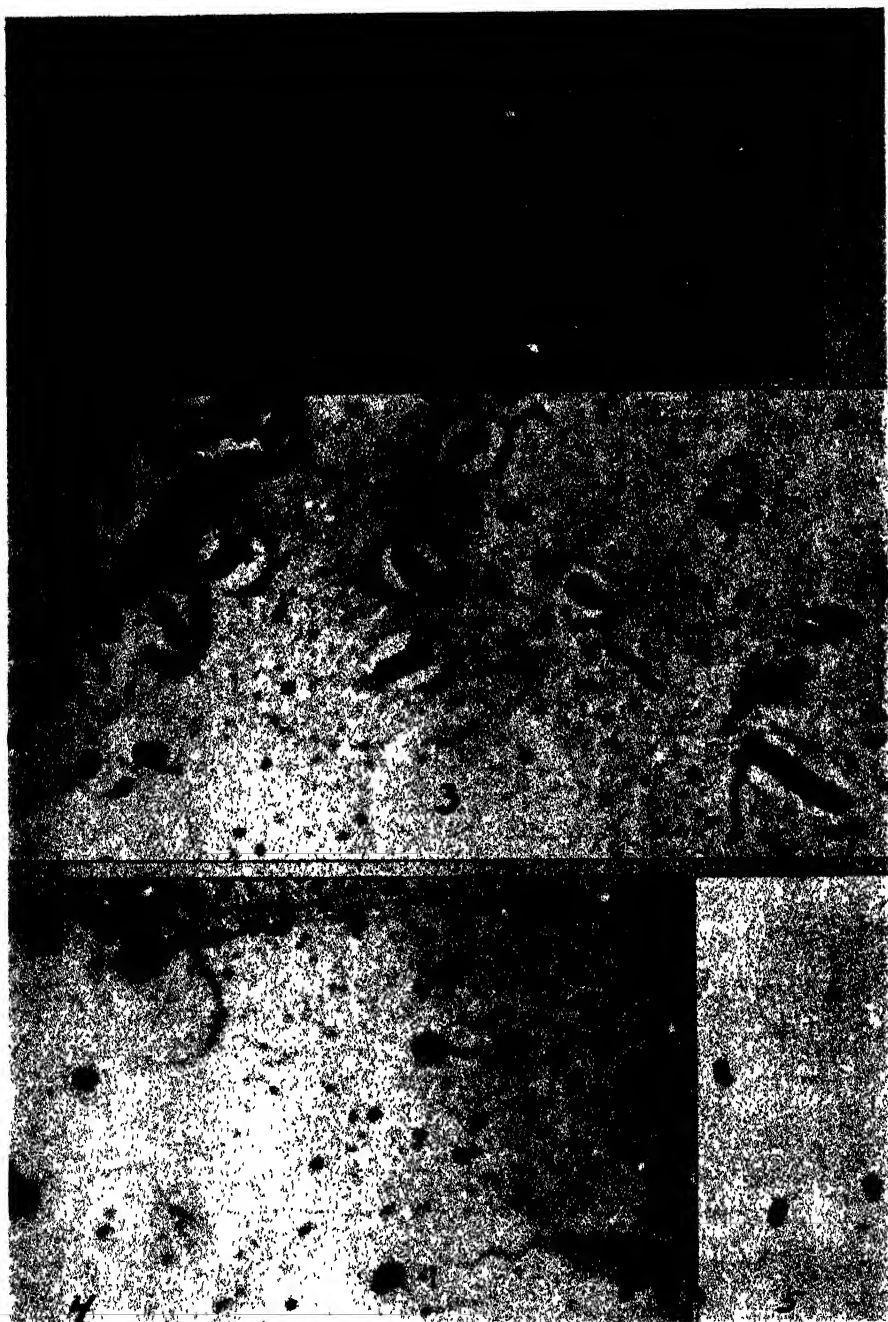
The cell of *Thiobacillus thiooxidans* consists of protoplasm surrounded by a thin cell-wall. It is heavily capsulated and provided with a flagellum. Depending on the form and size of the cell, the protoplasm may contain one, two, or several intraprotoplasmic structures, the nature and function of which will be discussed below.

a. The protoplasm. The protoplasm proper of *Thiobacillus thiooxidans* appears, both in bright and in dark-field, as a homogeneous system. Its volume is often greatly reduced due to the relatively large structures it includes. Its stainability with acid or basic dyes is similar to that of the protoplasts of other bacteria. It stains deeply with China blue (an acid dye) dissolved in $n\text{HCl}$, and with methylene blue dissolved in a phosphate buffer solution of pH 7; below pH 4, it stains with methylene blue only faintly. In this respect, the protoplasm of *Thiobacillus thiooxidans* behaves like those of the gram-negative bacteria (Stearn and Stearn, 1928).

b. The cell-wall and slime. Both the cell-wall and the slime were demonstrated by the method previously described (Knaysi, 1941). The cell-wall appears blue and the slime red. The cells are imbedded in the slime and held together *en masse* (fig. 2). The slime is probably secreted by the cell and is usually denser around the cells and appears, in smears, in the form of typical capsules. Typical capsulation can also be observed around single cells or pairs of cells which have broken away from the mass (figs. 1, 3 and 4).

In view of the extent of slime formation by *Thiobacillus thiooxidans* and of the ease with which that slime can be demonstrated, it appears surprising that no mention of it has been made by other workers. It is our belief that the "exceedingly faint halo", observed around the cells by Umbreit and Anderson (1942), and considered by them as an artifact, really represents the cell capsule. It is true that capsules are not usually demonstrated with the electron microscope when the cell suspension is prepared in the usual way. However, the work of Knaysi and Mudd (1942) on the pneumococcus shows that, when the cell suspension is prepared directly on the collodion film, the capsule of that organism can be readily observed. This procedure avoids the contact of the cells with relatively large volumes of water and probably preserves electron-scattering material occluded in the capsule. As Umbreit and Anderson are not specific about their technique, it is probable that they used liquid cultures followed by instantaneous washing which, in view of the nature of the capsule of *Thiobacillus thiooxidans*, was not sufficient to remove all material occluded in the capsule.

c. Motility and flagella. Waksman and Joffe (1922) described the organism as non-motile. However, Vogler and Umbreit (1941) explained the slight turbidity developed by the organism in liquid, sulfur-containing media, as due to



FIGS. 1, 3 AND 4. CELLS FROM A 4-DAY OLD CULTURE IN THE LIQUID, SULFUR MEDIUM, STAINED BY THE AUTHOR'S METHOD FOR THE DEMONSTRATION OF THE CELL-WALL

FIG. 2. CELLS FROM A 5-DAY OLD CULTURE ON THE THIOSULFATE AGAR MEDIUM, STAINED BY THE AUTHOR'S METHOD FOR DEMONSTRATING THE CELL-WALL

Note the poor contrast and definition and the cell masses due to excessive production of slime.

FIGS. 5 AND 6. CELLS FROM A 3-DAY OLD CULTURE, IN THE LIQUID, SULFUR MEDIUM, STAINED WITH METHYLENE BLUE AT pH 3.6-3.8



FIG. 7. CELLS FROM THE SAME CULTURE OF FIGURES 5 AND 6, STAINED WITH METHYLENE BLUE AT pH 1.6-1.8

FIG. 8. CELLS FROM A 5-DAY OLD CULTURE IN THE LIQUID, SULFUR MEDIUM, FIXED FOR 1 MIN. IN 95 PER CENT ALCOHOL, TREATED FOR 2 HRS. IN 0.02 PER CENT SODIUM BICARBONATE, AND STAINED WITH LUGOL'S SOLUTION

FIG. 9. CELLS FROM A 14-DAY OLD CULTURE ON THE THIOSULFATE AGAR MEDIUM, STAINED WITH LUGOL'S SOLUTION

Note the absence of granules stainable with iodine

FIG. 10. CELLS FROM A 15-DAY OLD CULTURE ON THIOSULFATE AGAR, STAINED BY A MODIFIED BURKE'S GRAM METHOD (OMISSION OF BICARBONATE; DECOLORIZATION WITH 95 PER CENT ALCOHOL FOR 10 SEC.)

Note the gram-positive vacuole in a gram-negative protoplasm

movement through the medium in search of sulfur. That implies their belief in the motility of the organism. Later, Umbreit and Anderson (1942) were able to observe an occasional flagellum with the electron microscope. However, the majority of the cells were non-flagellated.

In the present work, we have repeatedly observed the organism in dark-field, or in bright field stained by the method previously described (Knaysi, 1941) for the demonstration of the cell-wall. In dark-field, the majority of the cells are either non-motile or endowed with an extremely slow motion. Occasionally, however, one observes a cell with an extremely rapid motion. It is interesting to note that such cells are continuously shifting the direction of their motion, as if they were searching for something. This may be a justification for the hypothesis of Vogler and Umbreit outlined above. In stained preparations, one observes numerous free flagella and unflagellated cells (figs. 1, 3 and 4). There are, however, many cells provided with a single terminal, rarely lateral, flagellum. In capsulated cells, one often observes the flagellum throughout the capsule to its origin in the cell (figs. 1, 3 and 4). The lack of motility of many cells, and the extreme sluggishness with which many others move, is undoubtedly due to the fact that the majority of the cells are embedded in slime, for it has been shown (Knaysi, 1933) that a certain variant of *Bacillus megatherium* carried flagella but was non-motile because the cell chains were surrounded by a heavy capsule and grew in fascicles.

In the liquid, sulfur-containing medium, the flagellum is relatively thick, much thicker than on the solid medium. This confirms the early observations of Reichert (1909) on other bacteria. In the liquid culture, the thickness of the stained flagellum is about 0.15μ , about 0.15-0.19 of the cell width. With the exception of a few cases in which the flagellum is straight or bent into a loop, it is usually helicoidal in form and consists mostly of 5 turns. The width d of the helix is about 0.7μ and the altitude h of a turn is 1.3 to 1.8μ . This gives for the true length of a coil:

$$\begin{aligned}\lambda &= \sqrt{h^2 + \pi^2 d^2} = \sqrt{6.53} \text{ to } \sqrt{8.08} \mu \\ &= 2.6 \text{ to } 2.8\mu\end{aligned}$$

a flagellum of five coils would thus measure 13 to 14μ or, often, about 10 times the length of the cell.

d. Intra-protoplasmic structures. The electron micrographs published by Umbreit and Anderson (1942) show principally two types of intracellular structures: 1) an opaque mass occupying a large portion of the cell volume, 2) a helicoidal body present in some of the cylindrical cells and extending from one end of the cell to the other. A cell containing the helicoidal body may also contain one or more round bodies. The nature of those structures was not determined, but it was thought likely that they would consist of reserve material.

In the course of the present investigation, we made repeated attempts to redemonstrate these structures and determine, if possible, their nature and their function. We were particularly interested in those having a helicoidal form, because such structures were observed in some of the large bacteria (Swellen-

grebel, 1906; Dobell, 1911), and were considered as nuclei. Unfortunately, we have been so far unable to observe clearly any intracellular structure which could unmistakably be considered helicoidal; and we suspect that such forms are distinct in nature and in function from the ellipsoidal or spherical bodies regularly formed by the cell. Our study had, therefore, to be confined to the latter group.

Spherical or ellipsoidal intracellular bodies are formed by the majority of the cells; we believe that they may be potentially formed by all normal cells regardless of their form. Among the short, ellipsoidal cells (fig. 5), we noticed two groups; the first consists probably of resting cells containing each a single structure eccentrically located; the second consists of growing cells containing two such structures each, thus showing the "bipolar" appearance referred to by Umbreit and his co-workers. Among the elongated, cylindrical cells (figs. 6 and 7), we also distinguish two groups; the first includes relatively thick cells containing two (rarely one or three) terminal structures and probably consisting of resting cells; the second consists of more slender cells containing three or more structures each; when three structures are present, one is usually located in the center of the cell where partition is to be expected, and the other two are terminal. In young cultures growing on the thiosulfate agar medium, the majority of the cells are of this latter type.

In studying the *nature* of these intracellular structures, one is led to distinguish between those formed in the liquid, sulfur-containing medium and those formed on thiosulfate agar. In untreated cells, grown in the *liquid, sulfur medium*, the structures stain with methylene blue at pH 1.7, whether the cells were not fixed or whether they were fixed with heat, or with 95 per cent alcohol from 6 to 48 hours. They become unstainable with methylene blue at pH 1.7–4.0 after exposure of the cells to water at 80°C. for 10 minutes, to 0.02 per cent sodium bicarbonate for 2–3 hours, or when the cells were allowed to starve. Starvation was brought about by letting the culture stand from 16 to 24 hours after removal of the sulfur by filtration through paper. According to our present understanding, the above reactions indicate that the structures contain, or consist of, a reserve material having several of the properties of volutin (see Knaysi, 1942). This conclusion is strengthened by chemical analysis of the bicarbonate extract. According to Umbreit (1943), a solution of 0.20 per cent bicarbonate extracts, in 3 hours, 11.2 per cent of the organic phosphorus and 6.4 of the nitrogen of the cells. The extracted material also contains pentose in the molar ratios of 1/2.2/27 to phosphorus and nitrogen respectively. On the other hand, when the untreated cells are placed in contact with Lugol's solution (I_2 -KI), the structures assume a deep brown color characteristic of fat; they also stain to a variable extent with Sudan III. In cells fixed in 95 per cent alcohol for six hours, staining with iodine and with Sudan III was still apparently unimpaired in the majority of the cells. However, treatment of the cells with 0.02 per cent sodium bicarbonate reduced but did not destroy, stainability with iodine and with Sudan III. In the majority of the cells, the structures usually appeared somewhat disorganized and stained less definitely, often presenting only a stained contour; in certain cells and cultures, however, they can be clearly dem-

onstrated (fig. 8). Stainability with iodine is generally lost upon starvation of the cells. In cultures 6 to 15 days old grown on *thiosulfate agar*, the intracellular structures stain yellow-brown with iodine (fig. 9) and only faintly with Sudan III; they take up methylene blue at pH 1.7–4 in untreated cells, but not in cells exposed to 0.02 per cent sodium bicarbonate for 2–3 hours. Consequently, in cells grown on thiosulfate agar, the structures seem to contain, or consist of, a single substance having the properties of volutin.

The above reactions lead to the *conclusion* that the intraprotoplasmic structures observed in the cell of *Thiobacillus thiooxidans* and sometimes responsible for the "dipolar" staining of the cells are large vacuoles containing reserve material. The existence of a vacuolar membrane, probably lipoid-containing, can also be demonstrated. When the cells are grown in a liquid medium containing elementary sulfur, the content of the vacuole consists either of a new substance having several of the properties of volutin, and other properties different from those of volutin, or of an intimate mixture of volutin with another substance. If it is a mixture, the two substances must be so dispersed that the individual particles are not resolved with the microscope. The nature of the second substance, whether it be free or combined with volutin, needs discussion. Its behavior toward iodine and Sudan III tends to indicate a fatty nature. However, it is our experience that reserve fat is usually removed when the cells are fixed in 95 per cent alcohol for 48 hours. Taking into consideration the fact that volutin alone is formed when the cells are grown on the thiosulfate medium, one would suspect that, in the presence of elementary sulfur, the vacuole contains either elementary sulfur, or a sulfur compound of the indicated properties, associated with volutin. This suspicion is strengthened by the fact that, when minute particles of flowers of sulfur are placed in the well of a hanging drop slide and allowed to melt by passing the slide over the flame, those particles give, after resolidification, deep brown coloration with iodine, stain with Sudan III, and are not dissolved in 95 per cent alcohol. We are therefore inclined to conclude that the reserve material normally formed by *Thiobacillus thiooxidans* is volutin, and that, in the presence of elementary sulfur, some form of sulfur may also be present in the vacuole as a reserve material. This would explain Vogler's finding (1941) that, in the presence of sulfur, "the organism synthesizes a reserve storage product which enables it to live in the absence of oxidizable sulfur."

The gram reaction

The literature contains contradictory statements regarding the gram reaction of *Thiobacillus thiooxidans*. Waksman and Joffe (1922) stated that the organism is gram-positive. On the other hand, Starkey (1935) found it gram-negative. Umbreit, Vogel and Vogler (1942) explain the discrepancy by a pH difference in the staining solutions and its influence on the chemical reactivity of fat.

In the present investigation we first used Burke's method (1922) which usually gives more clear-cut results than most other techniques. For reasons which will soon become obvious, we finally modified that method by omitting the addi-

tion of sodium bicarbonate, by increasing the time of contact with iodine to 2 minutes and by decolorizing with 95 per cent ethyl alcohol for 10 seconds. Acetone could also be used for only a few seconds.

The results of our study with both the liquid and the solid cultures have shown that the protoplasm of *Thiobacillus thiooxidans* is definitely gram-negative, whereas the vacuolar content is gram-positive (fig. 10). Young cells which have not had time to accumulate reserve material, and cells which do not contain reserve material, either because it was used up or removed with mild alkalies, are gram-negative throughout. In young cells one can often discern a spot, corresponding to the vacuole, showing a border-line color because of the presence of a small amount of reserve material. In view of the relatively large size of the vacuole and of the fact that the cultures generally used are at a stage when the cells are rich in reserve material, we can very well see how it is possible to overlook, in ordinary microscopic observations, the often thin protoplasmic layer and to call the organism gram-positive. On the other hand, most dye solutions used in gram-staining contain an alkali to intensify the staining (mordant action of OH^-), and the alkalinity of those solutions may be sufficiently high to remove the volutin from the vacuole. This is particularly true in Burke's method. By this method, the cell appears gram-negative, with merely a gray or border-line color at the locus of the vacuole. Certain cells may even show an empty vacuole and a vacuolar membrane of doubtful reaction.

DISCUSSION

We hope that the observations reported in this paper will be instrumental in bringing about a solution to the question: How is *Thiobacillus thiooxidans* able to attack elementary sulfur? The answer given by Umbreit, Vogel and Vogler (1942) is that the organism "oxidises insoluble sulfur by dissolving it in a fat globule, located at the end of the cell". This fat droplet is considered to be the same structure which may, "under given staining procedures give the organism a 'dipolar' appearance". The later work of Umbreit and Anderson (1942) with the electron microscope, however, did not reveal the existence of such a globule. The present investigation shows that the structure responsible for the dipolar staining of the organism is intra-protoplasmic; it is a vacuole, containing reserve material. Regardless of the nature of that reserve material it is difficult to see how such an intra-protoplasmic structure can be placed in contact with the sulfur particles of the medium, especially when the cell is embedded in slime.

Another question which may be asked regarding *Thiobacillus thiooxidans* is: By what mechanism is the organism able to survive, and even to grow, at the low pH developed in its medium ($\text{pH} \leq 1$). The present investigation shows that the protoplasm of the organism is neither strongly acidic, nor do its constituents exist below their iso-electric point. It seems possible to us that the strongly acidic, relatively huge vacuole usually present in the cell, plays a role in the pH balance between the protoplasm and the exterior environment.

Finally, the results of the present investigation have a direct bearing on the theory of the gram reaction. They bring out the relation between the acidic

strength of a cell constituent and its gram reaction (Stearn and Stearn, 1928), and they show that a cellular structure may be gram-positive without being fatty in nature. Moreover, our observations on the young cells indicate that the results of the gram reaction may depend on the quantity of gram-positive material present in the cell; the larger is the quantity, the greater is the amount of dye bound and the greater is the mass of insoluble compound formed with the mordant; in the young cells the gram-positive material may be present, but its concentration is less than in older cells, and the final color is not so obvious under the microscope. Finally, the coexistence of both gram-positive and gram-negative structures within the same cell, is an indication that a difference in the gram reaction may exist without a corresponding difference in the permeability of the cell "membrane", and it is doubtful whether it can be attributed to the vacuolar membrane. In view of the brilliant work of Burke and Barnes (1929) it is not the author's intention to deny that a difference in permeability may exist between gram-positive and gram-negative cells. It can be pointed out, however, that, if such a difference exists, it would tend to intensify other qualitative differences already existent between the cells, for the gram reaction is probably not the result of a single factor, but an expression of the resultant of several inherent differences.

SUMMARY

The cell structure of *Thiobacillus thiooxidans* is fundamentally similar to that of other bacteria. It consists of a gram-negative protoplasm (pH of isoelectric point > 4) containing one or more large vacuoles. When the medium contains elementary sulfur, the vacuolar content gives the reactions of both volutin and sulfur; on Waksman's thiosulfate medium, only volutin is formed. The protoplasm is surrounded by a cell-wall, and the cells are imbedded in slime. The cells are actively motile and provided, each, with a single, thick terminal flagellum. The bearing of the present study on the mechanism of some of the physiological processes of the organism and on that of the gram reaction are discussed.

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THE PREPARATION OF A POLYVALENT DYSENTERY BACTERIOPHAGE IN A DRY AND STABLE FORM¹

I. PRELIMINARY INVESTIGATIONS AND GENERAL PROCEDURES

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The purpose of the present work was to investigate the conditions for the production of a polyvalent dysentery bacteriophage in addition to its concentration and conversion to a dry stable form. Throughout this study, emphasis was placed upon those procedures readily adapted to large scale operation.

Polyvalent bacteriophage preparations for therapeutic use in dysentery and cholera have been described by several authors (D'Herelle, 1922; Morison, 1930; Compton, 1929; Nahimson, 1942). The usual method of preparing a polyvalent phage lysate is to develop each of the constituent phage races against its susceptible strain of bacteria and then to mix mechanically the several lysates. A similar as well as a new procedure are described in this paper.

Preliminary to conversion of the polyvalent liquid phage to the dry state, it is desirable economically first to concentrate the lysates by vacuum distillation. Following Northrop (1939), who successfully concentrated staphylococcus phage lysates by this method to one-twentieth their original volume, we concentrated our polyvalent dysentery phage lysates ten to twenty times without incurring losses in titer.

For conversion of the vacuum-concentrated lysates to the dehydrated form, drying from the frozen state or "lyophilization" was employed (Flosdorf and Mudd, 1935). This method is commonly used for the preservation of such biological materials as bacteria, viruses, blood plasma, etc. Previous studies on the effects of freezing and drying on bacteriophage have been made by relatively few workers. These investigations are chiefly concerned with the possibilities of using the relative effects of these treatments on individual phage races for differentiation and classification purposes (Rivers, 1927; Knorr and Ruf, 1934-35; Colwell, 1937; Campbell-Renton, 1941; Krupin and Farafontova, 1942). In our work, lyophilization has been used primarily for the preparation of a stable, concentrated, and fully active phage material in a form especially suited to convenient therapeutic administration.*

EXPERIMENTAL—PREPARATION OF A POLYVALENT BACTERIOPHAGE

Materials

The strains of dysentery bacteria and the races of dysentery bacteriophage that are reported on in this communication are listed in table 1.

¹ The authors are greatly indebted to Mr. James S. Wallerstein, not only for constant encouragement and many helpful suggestions during the prosecution of the work reported in this paper, but also for making it possible.

Methods

Medium. In general, the choice of medium to be used for large scale production of bacteriophage should take into account the following factors: (a) stability for growth of the bacteria; (b) the enhancement of phage production; (c) stability of the produced phage; (d) proposed method of administration of phage for therapy; (e) ease of preparation; (f) cost. Further, if lyophilization of the lysate is envisaged, a low salt content is desirable. The following medium has proven very satisfactory in these respects and is to be recommended for the production of dysentery bacteriophage on a large scale:

500 grams of casein (technical), 500 grams of dried brewer's yeast, and 10 grams of dried pork pancreas (technical) are mixed in 7 liters of M/5 disodium phosphate. The mixture

TABLE 1
Dysentery bacteria and bacteriophages studied

SPECIES ^a	CODE NO. ^b	SOURCE OF ORGANISMS ^c	SOURCE OF PHAGES ^c
<i>Shigella dysenteriae</i>	SH-1	P.G.H. (Dubos) ^d	Hudson River
<i>Shigella paradysenteriae</i> (Flexner types)	F-2	P.G.H. (Dubos)	Hudson River
	F-3 (VZ)	P.G.H. (Dubos)	Hudson River
	F-5 (Z)	P.G.H. (Dubos)	Hudson River
<i>Shigella sonnei</i>	S-204	P.G.H. (Dubos)	Mixed coli phages
<i>Shigella</i> sp. (Newcastle type)	N-1	N. Y. C. Bd. of Health	Mixed coli phages

^a Nomenclature follows that found in Bergey's "Manual of Determinative Bacteriology." Fifth edition, 1939. Williams & Wilkins Co., Baltimore, Md.

^b Each bacteriophage has been given the code number of the susceptible bacterial strain against which it has been developed and propagated.

^c (P.G.H.) Post Graduate Hospital, through the courtesy of Dr. Ward J. MacNeal.

^d (Dubos) Transfers from Dr. Rene Dubos, Harvard University.

is adjusted to pH 7.5 with sodium hydroxide, put in a 12-liter flask with 10 ml. of toluene, shaken, brought to 37°C., and maintained at that temperature for 48 hours. The 48-hour hydrolysate is then autoclaved at 10 lbs. pressure for 30 minutes. After autoclaving, the hydrolysate is either filtered with the help of celite or, preferably, centrifuged in a Sharples centrifuge. The filtrate, readjusted to pH 7.4 with sodium hydroxide, is made up to a volume of 10 liters. (Biuret tests for unhydrolyzed protein are negative.) For use as medium, this hydrolysate is diluted ten times. (A Kjeldahl N. determination on a sample of the medium showed 0.45 gram of nitrogen, calculated as peptide nitrogen, per 100 ml.)

Preliminary tests were run with the yeast-casein hydrolysate to determine the suitability of this medium for phage preparation. Phages of high titer, equivalent to results obtained in meat extract broth, were produced.

Assay. Assay of the titers of the phage preparations has been made by means of the broth dilution method.

The phage preparation is diluted by serial dilution in 4.5 ml. of broth in tubes with the addition of 0.5 ml. of phage or phage dilution until a dilution of 1×10^{-11} is reached (11

tubes) with a fresh pipette for each transfer. Approximately 100–200 million organisms are added to each tube in 0.1 ml. of a broth suspension of an 18 hr. culture on a nutrient agar slant. The suspension is added first to a tube of broth, serving as organism control, and then added to phage dilutions proceeding from highest dilution to the lowest. Incubated at 37°C. the tubes are read for the extent of lysis in 24 and 48 hours; a preliminary reading is made after 4 hours. We have counted as effective lysis both complete clearing of the broth and clearing with barely perceptible growth of organisms when the tube is viewed obliquely. The phage assay is then reported as the highest dilution in which lysis has occurred.

Selection of phage races. Our primary interest in the present attempt to prepare a polyvalent phage mixture was to have each of our species of dysentery bacteria represented by phages capable of lysing all of the bacterial strains of a given species at hand rather than to make a mixture that might prove generally to be therapeutically effective.

Since the production of a polyvalent dysentery phage preparation demands some kind of mixing of a number of individual phage races developed against their susceptible bacterial strains, it is desirable to learn which phage races are active against the largest number of representatives of a given bacterial species. Choice of the phage races which together are capable of lysing all of the available dysentery bacteria reduces the number of phages and their susceptible bacteria that has to be employed in the preparation of a polyvalent phage. In order to determine which of our phages were capable of lysing the greatest number of strains of a given dysentery bacterial species, cross tests were used. The details of the method are as follows:

Approximately 100–200 million organisms, in 0.1 ml. of a broth suspension of an 18 hr. culture on a yeast casein agar slant incubated at 37°C. are added to 3 of 4 test tubes containing approximately 10 ml. of beef extract broth. One of the inoculated tubes is reserved as an organism control. 0.5 ml. portions of the phage preparation to be tested are added to the remaining two of the bacteria-inoculated tubes as well as to the fourth tube which serves as a phage sterility control. The tubes are shaken, incubated at 37°C., and read at 24 hours. Under these conditions, the standard of activity for the phage preparation was complete lysis.

Preparation of polyvalent phages. After deciding which phages should be included to make an effective polyvalent phage mixture on the basis of the cross tests as well as on the basis of stability through many transfers, two kinds of mixtures were devised.

One kind involved merely the mechanical mixing of 5-liter batches of each of six phages chosen.

In the preparation of the 6 constituent phages, 5 liters of yeast casein hydrolysate in a 9-liter Pyrex serum storage bottle were individually inoculated with 50 ml. of a bacterial suspension, (washings of 5 slants of our 18-hour cultures at 37°C.) containing approximately $3.5\text{--}4.0 \times 10^{10}$ organisms, and seeded immediately with 0.5 ml. of the stock phage containing 5×10^7 phage particles. These cultures were incubated for 48 hours at 37°C. in a constant temperature room. At the end of this time, they were filtered individually through sterile Hermann filters¹ #8. The filtrates were mechanically mixed by measuring equal quantities

¹ F. R. Hormann & Company, 127 Boerum Place, Brooklyn, N. Y.

of the 6 constituent phages into a large bottle. The mixture was then refiltered through Hormann filters #8, and stored in the icebox. Assays were done on the individual phages as well as on the mixtures. We have termed this type of mixture, the mechanically mixed phage, or M.M.P. for short hand designation.

The other type of mixture was accomplished by inoculating the final phage growth medium with appropriate amounts of 7 bacterial strains and by seeding it with their respective phage races.

5 liters of yeast casein hydrolysate in a 9-liter Pyrex serum storage bottle were inoculated with a suspension (washings of 7 slants of 18-hour cultures at 37°C) containing approximately 7×10^9 organisms of each of the 7 constituent susceptible bacterial strains. 3.6 ml. of each of the 7 stock phages were added immediately, giving 3.6×10^9 phage particles for each phage race. This culture was then incubated for 48 hours at 37°C. in a constant temperature room. At the end of this time, the phage was filtered through a Hormann #8 filter. The filtrate was assayed and stored in the icebox.

It should be noted that, in the preparation of this polyvalent phage mixture, the constitutive elements are kept as separate entities until the last stage of the operation has been reached. In consideration of the probable difficulties attendant upon large scale production of a polyvalent dysentery phage mixture, the advantages of this latter method should not be overlooked. We have termed this type of mixture the original mixed phage or O.M.P. for short-hand designation.

Results

Data on the stability through many transfers of the several phage materials supplied us, and a consideration of their effectiveness in cross reaction tests, led us to choose the following six phages to be included in the mechanically mixed phage preparation: SH-1, F-2, F-3, F-5, N-1, and S-204. Table 2 presents a compilation of the assays made on the titers of the six stock phages used for seeding of the 5-liter batches, the titers of the 5-liter batches themselves, and the titers of the component phages against their susceptible bacterial strains as present in the mechanically mixed preparation. The method as outlined above was used to produce the final phage mixture.

It is clear that satisfactory propagation of the phage is supported by this medium under the conditions given. As expected, no appreciable loss in titer of any of the phage constituents occurred through the mere process of mixing.

The lytic polyvalency of the M.M.P. was tested against 31 dysentery bacterial strains. These included 7 Shiga, 5 Sonne, 18 Flexner, and 1 Newcastle strains. The method used was the same as that described for the phage cross-reaction tests. Corresponding data were obtained for the activity of the M.M.P. after converting it into a dry powder through a method of lyophilization to be described below. The powdered lyophilized M.M.P. was dissolved in water to the original volume before determining its polyvalency. All of the strains, with the exception of one Sonne strain, are lysed by the phage mixture. We appreciate that in these tests relatively high concentrations of phage are used. However, the results, in confirming the cross-reaction tests, furnish evidence

that judicious choice of phage races to be included in a mixture can insure its polyvalency. Of additional interest is the comparable effectiveness of the lyophilized sample, a fact which supports the feasibility of this method for producing a stable, concentrated phage preparation.

While it is demonstrably possible to prepare a polyvalent phage mixture by mechanically combining volumes of individually developed phage races, the

TABLE 2
Data on the preparation of mechanically mixed phage
Assays with homologous organisms

STRAIN OF ORGANISM USED	TITER OF STOCK PHAGE USED TO SEED 5-LITER BATCHES		TITER OF PHAGE IN FILTRATE OF 5-LITER BATCHES		TITER OF PHAGE IN MECHANICALLY MIXED PHAGE		FACTOR OF INCREASE IN NO. OF PHAGE PARTICLES PER ML.
	24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.	
SH-1	10^{-7}	10^{-7}	10^{-8}	10^{-7}	10^{-8}	10^{-9}	10^4
F-2	10^{-9}	10^{-10}	10^{-10}	10^{-9}	10^{-10}	10^{-9}	10^4
F-3	10^{-10}	10^{-8}	10^{-9}	10^{-9}	10^{-8}	10^{-8}	10^5
F-5	10^{-4}	10^{-3}	10^{-9}	10^{-7}	10^{-9}	10^{-8}	10^5
N-1	10^{-8}	10^{-8}	10^{-9}	10^{-9}	10^{-8}	10^{-8}	10^6
S-204	10^{-8}	10^{-6}	10^{-8}	not read	10^{-7}	10^{-7}	10^6

The titer represents the highest dilution showing effective lysis (complete clearing or clearing with barely perceptible growth of organism).

TABLE 3
Data on preparation of original mixed phage

STRAIN OF ORGANISM USED	ASSAY OF PHAGE				FACTOR OF APPARENT INCREASE IN NUMBER OF PHAGE PARTICLES PER ML.
	Seed phage		O.M.P.		
	24 hr.	48 hr.	24 hr.	48 hr.	
SH-1	10 ⁻⁷	10 ⁻⁷	10 ⁻⁸	10 ⁻⁸	10 ⁵
F-2	10 ⁻⁹	10 ⁻⁹	10 ⁻¹⁰	10 ⁻⁹	10 ⁴
F-3	10 ⁻¹⁰	10 ⁻⁸	10 ⁻⁹	10 ⁻⁹	10 ⁵
F-5	10 ⁻⁴	10 ⁻³	10 ⁻⁸	10 ⁻⁹	10 ¹⁰
N-1	10 ⁻⁸	10 ⁻⁸	10 ⁻⁹	10 ⁻⁹	10 ⁶
S-203	10 ⁻⁹	10 ⁻⁸	10 ⁻⁶	10 ⁻¹⁰	10 ⁶
S-204	10 ⁻⁸	10 ⁻⁶	10 ⁻⁹	10 ⁻⁸	10 ⁶

The titer represents the highest dilution showing effective lysis (complete clearing or clearing with barely perceptible growth of organisms.)

practical difficulties associated with this method for large scale operation give added interest to the data given in table 3. Employing the method given above for the production of a polyvalent phage mixture by inoculating and seeding simultaneously the final culture medium with all of the selected susceptible bacterial strains, and their phages, we obtained the recorded results.

In the preparation of this original mixed phage, an additional phage was included, with its susceptible bacterial strain, S-203. Comparison of the factors

of apparent increase in the number of phage particles for each phage used in this mixture with those of the M.M.P. (cf. table 2) indicates that no obvious loss in titer of any of the constituent phages has taken place. It is recognized that some interaction of the several phages does occur which might mask such a loss in titer, but the assays suggest that the losses, if they occur at all, are of no practical significance.

As with the mechanically mixed phage, the original mixed preparation, before and after lyophilization, was tested for its polyvalency with all of the available stock cultures. 30 of the 31 strains were lysed as with the M.M.P.

Under the conditions of the tests, it is clear that the O.M.P. both in the liquid and dry form has a polyvalency equivalent to that shown by the M.M.P. These results give further supportive evidence that the method of preparing the

TABLE 4

Concentration of polyvalent dysentery bactiophage preparations by removal of water by vacuum distillation

520 ml. mechanically mixed polyvalent dysentery phage preparation (M.M.P.) concentrated to 53 ml. in glass vacuum still at a maximum bath temperature of 50°C. Duration of concentration process approximately 5 hours. Vacuum (Pressovac Pump) 35 mm. Hg. For assay, the concentrated material was filtered through a Jenkins bacterial filter.

STRAIN OF ORGANISM USED	TITER OF M.M.P. #1 PRIOR TO CONCENTRATION		TITER OF M.M.P. #1 AFTER CONCENTRATION TO 1/10 VOL.	
	24 hr.	48 hr.	24 hr.	48 hr.
SH-1	10 ⁻⁸	10 ⁻⁸	10 ⁻⁹	10 ⁻⁹
F-2	10 ⁻¹⁰	10 ⁻⁹	10 ⁻¹⁰	10 ⁻¹⁰
F-3	10 ⁻⁹	10 ⁻⁸	10 ⁻⁸	10 ⁻⁸
F-5	10 ⁻⁹	10 ⁻⁸	10 ⁻⁹	10 ⁻⁸
N-1	10 ⁻⁷	10 ⁻⁷	10 ⁻⁸	10 ⁻⁷
S-204	10 ⁻⁸	10 ⁻⁸	10 ⁻⁷	10 ⁻⁷

original mixed phage is a practicable one which warrants serious consideration for possible large scale polyvalent phage production.

CONCENTRATION AND DRYING OF POLYVALENT PHAGE MIXTURES

The advantages of a concentrated, dried bacteriophage preparation over the usual liquid lysates include greater stability, decrease in bulk, simpler means of administration, etc. Especially when the material is to be used for therapeutic purposes under a variety of sometimes extreme conditions, it is desirable to have the phage immediately available in a stable and active form.

As a preliminary step in the preparation of such phage material the polyvalent lysate mixture (M.M.P.) can be concentrated to at least one-tenth its volume in a conventional glass vacuum still without significant loss in titer. During the concentration, the temperature of the lysate is not permitted to rise above 35°C., while the maximum bath temperature is kept at 50°C. Table 4 presents data obtained when a sample of M.M.P. was concentrated approximately ten times.

Considering it desirable to remove as much salt from the lysate as is conveniently possible in order to decrease the anticipated hygroscopicity of the dried phage preparation, we dialyzed a sample of M.M.P. in cellophane tubing

TABLE 5

Concentration of M.M.P. #1 by removal of water by vacuum distillation after dialysis

Arrangement the same as in experiment described in table 4 except that the bacteriophage preparation was dialyzed through cellophane tubing against running tap water (22.5°C.) for 17 hours. During dialysis, the volume of the phage preparation increased from 520 ml. to 570 ml. The dialyzed solution was concentrated in vacuo to 34 ml.

STRAIN OF ORGANISM USED	TITER OF M.M.P. #1 PRIOR TO CONCENTRATION		TITER OF M.M.P. #1 AFTER DIALYSIS		TITER OF M.M.P. #1 AFTER DIALYSIS AND SUBSEQUENT CONCENTRATION TO 1/10 VOL.	
	24 hr.	48 hr.	24 hr.	48 hr.	24 hr.	48 hr.
SH-1	10^{-8}	10^{-8}	10^{-7}	10^{-8}	10^{-9}	10^{-8}
F-2	10^{-10}	10^{-9}	10^{-9}	10^{-9}	10^{-11}	10^{-9}
F-3	10^{-9}	10^{-8}	10^{-7}	10^{-7}	10^{-10}	10^{-8}
F-5	10^{-9}	10^{-8}	10^{-8}	10^{-9}	10^{-10}	10^{-9}
N-1	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-8}	10^{-8}
S-204	10^{-8}	10^{-8}	10^{-9}	10^{-9}	10^{-9}	10^{-7}

TABLE 6

Analysis of some data in table 5 to indicate instability of lysis with the dialyzed M.M.P. #1 when assayed with two susceptible strains of organisms

DILUTION OF PHAGE	ASSAY WITH F-5		ASSAY WITH S-204	
	Original	Dialyzed	Original	Dialyzed
10^{-1}	4+ ^a	4+	4+	4+
10^{-2}	4+	4+	4+	4+
10^{-3}	4+	4+	4+	4+
10^{-4}	4+	4+	4+	4+
10^{-5}	4+	2+ ^b	4+	2+
10^{-6}	4+	2+	4+	2+
10^{-7}	4+	2+	4+	0
10^{-8}	4+	2+	4+	0
10^{-9}	1+ ^c	4+	0	4+
10^{-10}	0 ^d	0	0	0
10^{-11}	0	0	0	0

^a 4 indicates complete lysis.

^b 2 indicates definite lysis with bacterial turbidity present.

^c 1 indicates less turbidity than in control tube of organisms without phage.

^d 0 indicates absence of lysis.

against running tap water for 17 hours. Table 5 summarizes results which show that dialysis does not significantly affect the titer of the constituent phage races when mere vacuum distillation is used for concentration.

However, some irregularity with respect to complete lysis throughout the dilution series of a titration was noted with several of the constituent phage

ances. Since such irregularity was not evident in the titrations of the M.M.P. without dialysis, it appeared possible that some instability of the lytic power of the phage was occasioned by the dialyzing procedure. For example, table 6 illustrates the case for two of the constituent phage races.

LYOPHILIZATION³

After having concentrated our polyvalent dysentery phage mixtures M.M.P. and O.M.P. approximately ten times by vacuum distillation, we sought to convert them into a wholly dried form. For this purpose we chose the method of drying from the frozen state as being the most desirable since experience has shown that in general the activity of biological materials as well as their solubility are best preserved by this method. Drying from the frozen state, commonly termed "lyophilizing" (Flosdorf and Mudd, 1935) consists essentially in the removal of water by sublimation from the frozen material under high vacuum. The water thus removed can be condensed on very cold surfaces or it may be absorbed by chemical drying agents or adsorbents (calcium sulfate, silica gel, etc.).

The lyophile apparatus used by us is of all glass construction, similar to the miniature unit described by Flosdorf and Mudd (1935) with minor modifications suggested by Dr. Crecelius, formerly of the Department of Bacteriology, Yale University. The conduit leading to the condenser is equipped with a standard taper glass joint to which suitable connecting pieces for the attachment of small glass ampoules or of round bottom and cylindrical flasks of 250 to 500 ml. volume can be joined. During the drying period, the condenser and a small trap are immersed in a dry ice-cellosolve mixture contained in a Dewar flask while a high vacuum is maintained by a Welch Duoseal motor-driven oil pump running continuously throughout the experiment. From time to time, the approximate degree of vacuum in the apparatus is checked with the aid of an externally applied spark tester. The phage material to be dried is pre-frozen on the wall of the drying vessel by rotating it in a dry ice-cellosolve bath until cracks appear in the solid layer thus formed. The lysate is distributed as uniformly as possible over the wall surface of the container to facilitate the passage of water vapors to the condensing system. For the experiments reported in this paper, volumes of lysates ranging from 60 to 70 ml. were dried from the frozen state in layers having an average thickness of 2.5 mm. We have, however, in another connection, successfully lyophilized frozen phage lysate from a layer at least 25 mm. thick.

EXPERIMENTAL

Before lyophilizing the ten-times-concentrated polyvalent phage lysate, we determined the stability of the unconcentrated M.M.P. when dried from the frozen state before and after dialysis. The conditions of the experiment and the results obtained are summarized in table 7.

Examination of the data discloses two principal facts. 1) With the exception

³ We wish to acknowledge our indebtedness to Dr. Kurt G. Stern for his stimulating co-operation regarding the problem of lyophilization of the phage.

of SH-1, the polyvalent phage mixture, M.M.P., can be converted to the dry state without significant loss in titer of the constituent phage races. The importance of the loss in titer of SH-1 phage rests in the possibility that other as yet un-investigated phages will also be inactivated by lyophilization under similar conditions. This loss in titer has been a matter for extended study on which we intend to report in the immediate future but suffice it to say that means have been discovered to protect this phage fully during the drying process.

2) When the lysate mixture is dialyzed and then lyophilized, significant losses in the titer of all the phage races occur. That dialysis of the lysate results in general instability of the polyvalent dysentery phage mixture suggests that some dialyzable material exerts a protective action on the lytic agents so that they can be dried without appreciable destruction. It now appears likely that the difference in behavior during lyophilization of the Shiga-1 phage when

TABLE 7

Lyophilization of M.M.P. #1 before and after dialysis

a) 70 ml. polyvalent, mechanically mixed dysentery phage preparation in yeast-casein medium (M.M.P.) dialyzed for 15.5 hours against running tap water (25–26°C.). Dialyzed solution prefrozen and dried in cylindrical container on small lyophile apparatus for 6 hours.

b) 66 ml. of the same phage preparation lyophilized without prior dialysis. Left attached to lyophile apparatus for 15.5 hours.

STRAIN OF ORGANISM USED	CONTROL—NOT LYOPHILIZED		LYOPHILIZED DIRECTLY		LYOPHILIZED AFTER DIALYSIS	
	24 hr.	48 hr.	24 hr.	48 hr.	24 hr.	48 hr.
SH-1	10^{-8}	10^{-8}	10^{-2}	10^{-2}	10^{-1}	10^{-1}
F-2	10^{-10}	10^{-9}	10^{-9}	10^{-6}	10^{-8}	10^{-3}
F-3	10^{-9}	10^{-8}	10^{-7}	10^{-7}	10^{-4}	10^{-3}
F-5	10^{-8}	10^{-8}	10^{-7}	10^{-7}	10^{-6}	10^{-2}
N-1	10^{-7}	10^{-7}	10^{-6}	10^{-6}	10^{-3}	10^{-2}
S-204	10^{-7}	10^{-7}	10^{-6}	10^{-6}	10^{-3}	10^{-2}

compared with the others included in the mixture relates to the quantitative difference in its requirement for the dialyzable factor or group of factors responsible for protection against the destructive effects of desiccation.

Lyophilizations of the ten-times vacuum-concentrated polyvalent phage mixtures M.M.P. and O.M.P. were successfully accomplished. The conditions of the experiments and the data accumulated are given in tables 8 and 9.

It will be noted that in these lyophilizations the flasks containing the frozen lysates were kept immersed in ice-salt mixtures rather than being exposed to the air. Under such conditions it was thought that the stability of the constituent phage races during drying would be enhanced, but subsequent experiment showed that no advantage of this kind is gained since similarly successful lyophilizations of the concentrated phage mixtures were realized when the drying flasks were exposed to room temperatures. Further, the latter lyophilizations were completed in approximately one-fifth of the time required when an ice-salt mixture was used.

The lyophilized polyvalent dysentery phage mixtures, when prepared according to the method herein outlined, readily form a creamy fluffy powder which can be admixed with fillers such as lactose or calcium carbonate to give a product

TABLE 8

Lyophilization of vacuum-concentrated polyvalent dysentery phage preparation at low temperature

60 ml. mechanically mixed polyvalent dysentery phage preparation (in yeast-casein digest medium) corresponding to 600 ml. original lysate prefrozen on walls of cylindrical flask and lyophilized in small apparatus. Flask held immersed in ice-salt mixture throughout the drying process lasting 48 hours. The temperature readings in the freezing mixture ranged from -21° to -11.5°C . (measured in upper part of Dewar flask). Dried material was fluffy in texture and completely solid even after warming up to room temperature. For assay, the material was dissolved to make a final volume of 60 ml. As control, a sample of the non-lyophilized vacuum concentrate was used.

STRAIN OF ORGANISM	CONTROL LIQUID (CONCENTRATED) M.M.P. #1		LYOPHILIZED CONCENTRATE	
	24 hr.	48 hr.	24 hr.	48 hr.
SH-1	10^{-9}	10^{-9}	10^{-6}	10^{-6}
F-2	10^{-10}	10^{-10}	10^{-9}	10^{-9}
F-3	10^{-8}	10^{-8}	10^{-8}	10^{-9}
F-5	10^{-9}	10^{-8}	10^{-11}	10^{-7}
N-1	10^{-8}	10^{-8}	10^{-8}	10^{-8}
S-204	10^{-7}	10^{-7}	10^{-7}	10^{-7}

TABLE 9

Lyophilization of O.M.P. #1 at low temperature after vacuum concentration

50 ml. of vacuum concentrated polyvalent mixed grown dysentery phage preparation (O.M.P.) corresponding to approximately 450 ml. of original lysate (yeast-casein digest medium) pre-frozen on the wall of 500 ml. round-bottom flask. The latter was attached to a small lyophile apparatus and held submersed in an ice-salt freezing mixture during the drying process, lasting 48.5 hours. The temperature recorded in the upper part of the freezing mixture ranged from -20° to -10°C . For assay, the dried material was dissolved in water to the volume of the vacuum concentrate. The latter served as control.

STRAIN OF ORGANISM USED	CONTROL LIQUID (CONCENTRATED) O.M.P.		LYOPHILIZED CONCENTRATE	
	24 hr.	48 hr.	24 hr.	48 hr.
SH-1	10^{-9}	10^{-9}	10^{-6}	10^{-6}
F-2	10^{-11}	10^{-11}	10^{-10}	10^{-9}
F-3	10^{-10}	10^{-8}	10^{-10}	10^{-9}
F-5	10^{-10}	10^{-11}	10^{-7}	10^{-7}
N-1	10^{-6}	10^{-8}	10^{-10}	10^{-6}
S-204	10^{-11}	10^{-9}	10^{-7}	10^{-7}

that can easily be pressed into pill form. By varying the amount of lyophilized phage in the lactose-phage mixture as well as by varying the size of the pill, any dosage that may be desired in one pill for therapeutic purposes can be realized. Details of tests run on the stability of the phage in the dry form will

be reported in a later communication. In general, however, it can be stated that if the phage powder is kept dry, no loss in activity is detectable after a period of six months at 37°C.

From the data reported in this paper on the production of polyvalent dysentery bacteriophage mixtures and their successful conversion into a dry, stable, concentrated form, there is every likelihood that similar treatment of staphylococcus, cholera, and coli phages, among others, would assure comparable and useful results.

SUMMARY

1. Procedures for the preparation of polyvalent dysentery bacteriophage mixtures are outlined with a view to production on a large scale. The choice of phage races to be included in the mixture, the medium considered most generally suitable, the details for developing large quantities of lysate, and the two methods of mixing employed, are discussed.

2. Concentration of either the mechanically or the originally mixed phage lysates by distillation under vacuum to $\frac{1}{10}$ to $\frac{1}{20}$ of their initial volume can be achieved without loss in titer of the constituent phage races.

3. By drying the mixtures of the dysentery bacteriophage lysates from the frozen state, concentrated, stable, and fully active phage preparations can be produced. Among the several phage races included in our polyvalent mixtures only the Shiga phage showed marked sensitivity to lyophilization.

4. Applicability of the procedure herein described to the production of concentrated, stable, and therapeutically useful staphylococcus, cholera, and coli phages, among others, is suggested.

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FIBRINOLYSINS FROM GAS GANGRENE ANAEROBES¹

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In an earlier paper, Reed, Orr and Smith (1941), demonstrated that the more actively pathogenic species of gas-gangrene anaerobes produce fibrinolysis when tested by the familiar procedure of Tillet and Garner (1933). Carlsen (1939) has previously shown that *Clostridium histolyticum* produces an active fibrinolysin. The eleven species of the genus *Clostridium* (some seventy-seven strains) tested fall into two distinct groups: a majority of cultures of *C. welchii*, *C. novyi*, *C. septicum*, *C. sordellii*, *C. chauvoei*, *C. histolyticum*, *C. sporogenes* and *C. tyrosinogenes* produce fibrinolysis of coagulated plasma from man, guinea pig, rabbit (except *C. sordellii*) and sheep (except *C. welchii*). In contrast no cultures of *C. fallax*, *C. tertium*, *C. aerofetidum* produce measurable fibrinolysis of human, guinea pig, rabbit or sheep plasma.

In the case of human plasma, *C. welchii* produced the most active fibrinolysis; 83% of twenty-six strains produced complete solution in 24 hours or less, 17% produced complete solution in 7 hours or less and a few in 1 to 2 hours. *C. histolyticum* cultures were about equally active. All the other active species required from 7 to 24 hours to complete solution of coagulated human plasma. For the most part, guinea pigs' and rabbits' plasma was more rapidly, and sheep plasma less rapidly, broken down than plasma from man by all the fibrinolytically active species of *Clostridium*. In this respect the fibrinolysins of clostridia resemble those of staphylococci (Madison and Dart, 1936; and Meter, 1937), and stand in sharp contrast to the fibrinolysin of *Streptococcus haemolyticus* (Le Mar and Gunderson, 1940).

In the following experiments, as well as those summarized above, all organisms were grown in chopped meat medium and tests for fibrinolysis, anticlotting or clotting factors made after 18 to 20 hours' incubation. Cultures in Brewer's medium or peptone-thioglycollate medium (Reed and Orr, 1942) generally gave reactions the same as parallel cultures in chopped meat, but after several generations in the thioglycollate-containing media results were more irregular.

1. *C. tetani*. In sharp contrast with the fibrinolytic activity of the toxigenic gas-gangrene species of *Clostridium*, five strains of *C. tetani*, all active toxin-producing strains, failed to produce any fibrinolytic activity when tested by the same methods used with the gas-gangrene species. It seems probable that this difference is an important factor in the manner of spread of the two groups of species in the animal body.

2. *Nature of fibrinolytic enzyme of Clostridia*. The species distribution of fibrinolytic activity does not suggest any relationship between this action and proteolysis. Garner and Tillet (1934) have shown that the fibrinolysin of haemolytic *Streptococcus* will resist heating to 100°C. for 60 minutes and that the

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heated substance does not contain proteolytic enzymes. The following experiments indicate that the same is true of the fibrinolysin from several species of *Clostridium*.

The procedure followed was essentially that of Garner and Tillet (1934). Actively fibrinolytic strains of *C. welchii*, *C. novyi*, *C. sordellii*, *C. septicum*, *C. sporogenes*, *C. histolyticum* and, for comparison *Streptococcus haemolyticus* Group A, were grown for 18 hours in chopped meat medium. Supernatants from all the cultures were tested for fibrinolytic action against rabbit plasma, by the previously described procedure. Other portions of the same cultures were heated to boiling for 5, 40 and 80 minutes and tested in the same manner. Seitz filtrates from part of the cultures were similarly tested before heating and after 5 minutes' boiling.

TABLE 1

Fibrinolysis by whole cultures of Streptococcus haemolyticus and six species of Clostridium, before and after boiling for 5 to 80 minutes

	PER CENT OF TESTS SHOWING SOLUTION OF CLOT IN							
	7 hours or less				7 to 24 hours			
	Un-heated	Boiled 5 minutes	Boiled 40 minutes	Boiled 80 minutes	Un-heated	Boiled 5 minutes	Boiled 40 minutes	Boiled 80 minutes
Whole cultures:								
<i>S. haemolyticus</i>	93	100	83	40	100	100	100	50
<i>C. welchii</i>	64	64	50	33	64	73	75	43
<i>C. novyi</i>	86	40	8	0	100	53	45	0
<i>C. sordellii</i>	60	42	25	0	70	66	42	17
<i>C. septicum</i>	60	50	50	46	83	66	66	46
<i>C. sporogenes</i>	51	25	25	0	58	58	50	0
<i>C. histolyticum</i>	100	50	12	0	100	72	18	0
Filtrates:								
<i>C. sporogenes</i>	92	46			100	77		
<i>C. histolyticum</i>	77	69			85	76		

The results summarized in table 1 clearly indicate that the fibrinolytic substance of *Streptococcus*, as previously observed by Garner and Tillet, withstands 40 minutes' boiling and, in approximately half the trials, withstands 80 minutes' boiling. It is also apparent that the fibrinolysin from *C. welchii* and *C. septicum* possesses approximately the same degree of thermoresistance as that of *Streptococcus* while those of the other four species of *Clostridium* are slightly less resistant. Bacteria-free filtrates of *C. sporogenes* and *C. histolyticum* are as active in fibrinolytic action as the whole cultures and they are active after 5 minutes' boiling.

Samples of the same whole cultures, unheated and heated, and filtrates, unheated and heated, were tested for proteolytic action. As a test solution, 20 grams of gelatin and 5 grams Na_2HPO_4 were dissolved in 1000 ml. of water and autoclaved in 20 ml. amounts. Ten ml. amounts of the unheated and heated cultures or culture filtrates were added to 20 ml. amounts of gelatin solution. Formol titrations for amino nitrogen were made immediately after mixing and

after 5 and 24 hours' incubation at 37°C. The results are summarized in table 2. It is apparent that neither the whole culture of *Streptococcus* or the filtrate produced a significant amount of hydrolysis of the gelatin under the experimental conditions, notwithstanding the fact that the same cultures produced active fibrinolysin. Of the six whole cultures of *Clostridium* tested, three, *C. novyi*, *C. sporogenes* and *C. histolyticum*, actively hydrolyze gelatin. It is, however, apparent that this proteolytic enzyme is completely inactivated by 5 minutes' boiling. As indicated in the preceding section, the fibrinolysins from these cultures are active after 40 minutes' boiling. Of the several culture filtrates tested only those from *C. histolyticum* show any measurable digestion of gelatin by the method used. Again it is apparent that the proteolytic enzyme is inactivated by 5 minutes' boiling while the fibrinolysin retained its activity.

TABLE 2

Hydrolysis of gelatin by unheated and heated cultures of Streptococcus haemolyticus and six species of Clostridium

	AMINO NITROGEN PER 100 ML.				
	At start	5 hours' incubation		24 hours' incubation	
		Unheated	Boiled 5 minutes	Unheated	Boiled 5 minutes
	mg.	mg.	mg.	mg.	mg.
Whole culture:					
<i>Streptococcus</i>	138	142		140	
<i>C. welchii</i>	255	226		251	
<i>C. novyi</i>	312	329	310	553	308
<i>C. sordellii</i>	339	349		290	
<i>C. septicum</i>	215	200		189	
<i>C. sporogenes</i>	262	318	286	619	277
<i>C. histolyticum</i>	536	840	473	1017	424
Culture filtrates:					
<i>C. histolyticum</i>	387	503	439	574	456

It is therefore apparent that the fibrinolysin from several species of *Clostridium*, like that from haemolytic *Streptococcus*, is thermostabile and unrelated to proteolytic enzymes of the cultures.

3. *Anti-fibrinolytic substance.* Tillet and Garner (1933) have shown that plasma clots from the blood of patients recovered from haemolytic *Streptococcus* infections are often completely resistant to fibrinolysis by active cultures of *Streptococcus*. Apparently an anti-fibrinolytic factor appeared in the blood.

A series of rabbits which had been immunized with toxins or with toxoids of *C. septicum* and *C. sordellii* until the blood exhibited a high titre of antitoxin (Reed and Orr, 1942), were tested for anti-fibrinolysins. Plasma from these animals and from normal rabbits was tested for fibrinolysis in the usual manner.

C. septicum cultures (the strain from which the toxin and toxoid was prepared) produced fibrinolysis of coagulated plasma from normal and *C. septicum* immune animals at approximately the same rate. *C. sordellii* cultures broke down rabbit plasma clots very slowly, as previously noted, but there was no significant differ-

ence between the rate of fibrinolysis of normal rabbit plasma and the plasma of specifically immunized animals. Similarly, coagulated plasma of these *C. septicum* and *C. sordellii* immunized animals and that of normal animals was broken down at approximately equal rates by cultures of other clostridia and streptococci.

There is therefore no evidence that immunization with toxin or toxoid results in the development of specific or non-specific anti-fibrinolytic factors in the blood of the immunized animal.

Neter (1937) has shown that *Staphylococcus* fibrinolysin may be neutralized *in vitro* by specific antiserum. *C. welchii*, *C. septicum* and *C. sordellii* antitoxins prepared in rabbits, unconcentrated, and *C. welchii* antitoxin prepared in horses, were mixed with cultures of homologous and heterologous organisms in various proportions, from equal parts of each, and incubated at 37°C. for one-half hour. Normal rabbit plasma and CaCl₂ were then added to make the usual fibrinolysis test proportions. The rate of fibrinolysis was not significantly affected by the antiserum.

It is therefore evident that we have been unable to reproduce with clostridia evidence of specific anti-fibrinolytic action as demonstrated for haemolytic streptococci. It is possible that anti-bacterial serum might have been more effective than antitoxin.

4. *Anti-coagulation factor.* Denis and Adham (1937), Tillet (1937), Christensen (1940) and others have observed that certain strains of haemolytic *Streptococcus* contain a factor which prevents coagulation of oxylated plasma by calcium. Neter (1937) has demonstrated the anti-clotting factor in cultures of *Staphylococcus*. A similar situation was encountered with several cultures of *Clostridium*. With *C. welchii*, in chopped meat media eight cultures, and in Brewer's broth with 0.2 per cent glucose, six cultures out of thirty-three tested prevented CaCl₂ from clotting rabbit plasma. On repeated trials about the same proportion of strains exhibited the anti-clotting factor but they were not always the same strains. In other words anti-clotting is a variable factor in these cultures. About the same proportion of cultures of *C. novyi*, *C. septicum*, *C. sporogenes* and *C. histolyticum* exhibited the anti-clotting and, as in the case of *C. welchii*, this proved to be a variable factor.

The anti-clotting factor was less active against guinea pig or human plasma than against rabbit plasma.

Tillet (1937) points out that the critical pH for the clotting of plasma is 5.0 to 5.5. It therefore follows that if the culture is sufficiently acid to bring the plasma-culture mixture to a more acid reaction, clotting will be prevented. On the other hand Denis and Adham (1937) consider that the anti-clotting factor is lactic or other organic acid and not directly related to the pH. In these experiments cultures of *C. welchii* and other clostridia containing the anti-clotting factor were, at the time of testing, never more acid than pH 6.0 and in most instances from pH 6.2 to 6.4.

5. *Clotting factor.* The familiar association of plasma clotting with pathogenicity of *Staphylococcus aureus*, Fisher (1936), suggested possibilities in the differen-

tiation of gas gangrene species. Some twenty-five cultures belonging to the six most important gas gangrene species: *C. welchii*, *C. septicum*, *C. novyi*, *C. sordellii*, *C. sporogenes* and *C. histolyticum* were tested for ability to clot guinea pig plasma by the methods ordinarily employed with *Staphylococcus* cultures. All cultures of the first four species were known to be pathogenic for guinea pigs (Reed and Orr, 1941, 1942). The two latter species were of doubtful pathogenicity. Two of five cultures of *C. novyi* and one of five cultures of *C. septicum* regularly produced rapid clotting; *C. histolyticum* and *C. sporogenes* produced slow clotting. Other cultures failed to produce clotting. It is therefore apparent that plasma-clotting is not a significant characteristic of this group of species.

6. *Fibrinolysin in gas gangrene wounds.* Fibrinolysins have been found in human tissues infected with *Streptococcus* by Neter and Witebsky (1936), Neter (1936), Neter and Young (1937) and in *Staphylococcus*-infected tissues by Neter (1937).

TABLE 3

Fibrinolysis by extracts of guinea pigs' muscle infected with gas gangrene

MUSCLE INFECTED WITH	AVERAGE TIME FOR FIBRINOLYSIS		
	Muscle extract		
	Undiluted	1-10	1-100
	<i>hours</i>	<i>hours</i>	<i>hours</i>
<i>C. septicum</i>	5	5	15
<i>C. welchii</i>	12	13	24
<i>C. novyi</i>	15	30	nil
<i>C. sordellii</i>	24	nil	nil
Normal muscle	nil	nil	nil

A series of guinea pigs infected in experimental wounds in the thigh with the principal gas gangrene species (Reed and Orr, 1941, 1942) and moribund from the infections were killed and the infected muscle examined for fibrinolysin. At the time of examination the infections had spread through the wounded leg and generally along the body wall to the axilla or further. The musculature of the wounded leg was macerated, extracted for an hour with an equal weight of saline and centrifuged. The supernatant fluid, undiluted and diluted 1 to 10 and 1 to 100 with saline, was examined in essentially the same manner as cultures: 0.8 ml. of muscle extract, 0.2 ml. of oxalated guinea pig plasma, 0.5 ml. of saline and 0.3 ml. of CaCl_2 solution, were mixed and incubated.

The results are summarized in table 3. It is apparent that the *C. septicum* infected muscle contains an active fibrinolysin. The active substance is not present in normal muscle.

Tests were also made for the possibility of an anti-fibrinolytic substance in normal or infected muscle. The introduction of such muscle extracts, as just described, to plasma-culture mixtures, just before CaCl_2 coagulation, had no significant influence on the rate of fibrinolysis by the culture enzyme.

Since the fibrinolysin is in measurable concentration in the musculature of the infected leg as a whole it is probably more concentrated in the heavily infected wound area and probably constitutes a factor favourable to the spread of the infection.

SUMMARY

1. It has been shown that all pathogenic species of the gas gangrene group and some non-pathogenic species produce active fibrinolytic enzymes.

2. *Clostridium tetani* does not produce fibrinolysis.

3. The fibrinolytic enzyme of clostridia, like that of haemolytic streptococci, is shown to be thermostabile and therefore independent of proteolytic enzymes produced by the organisms.

4. It has not been possible to demonstrate anti-fibrinolytic factors in specific antitoxins of the clostridia.

5. Plasma clotting is not a characteristic of the gas gangrene clostridia

6. The presence of fibrinolytic enzyme in gas gangrene wounds suggests that it is a factor in the spread of infection.

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NOTES

TWO NEW SALMONELLA SEROTYPES ISOLATED FROM MAN

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Two organisms, isolated in the laboratory of the Department of Pediatrics of the School of Medicine of Yale University, from children hospitalized at the New Haven Hospital were submitted to the Bureau of Laboratories of the Connecticut State Department of Health for classification. These proved to be hitherto undescribed, diphasic *Salmonella* serotypes differing from similar species previously described in the flagellar antigens of phase 2. As has been the custom, it seems desirable to afford these equal rank with existing types, at least until the suitability of specific designations based upon relatively slight serologic differences in this group has been settled.

The names suggested have been chosen with the idea that they may serve to direct the attention of physicians and public health workers to the importance of the salmonellosis of children. The original sources of these infections have not been ascertained. It is compatible with our knowledge of the group to assume that an animal reservoir will eventually be found. Hence, the names chosen may bear no relationship to the host distribution.

Through the kindness of Doctor Paul L. Boisvert and his co-workers case histories have been made available and are summarized below, together with the type descriptions.

1. *Salmonella infantis* n. sp.—This organism was first isolated from the blood culture of a 4-month infant girl; subsequently it was isolated repeatedly from stool cultures. The infant had been hospitalized with diarrhea and mild fever associated with asthmatic bronchitis.

Morphological and biochemical reactions of the organism were typical of *Salmonella*. The organism was indistinguishable from *S. virchow* except by antigenic analysis. Serological study including mirror adsorption tests showed the antigenic formula to be: VI₁, VI₂, VII: r—1,5

2. *Salmonella pueris* n. sp.—This organism was isolated from anal swabbings and stools of a 14-year boy during the course of gastro-enteritis complicating measles.

Morphological and biochemical reactions of the organism were indistinguishable from *S. newport* from which it could be differentiated only by mirror adsorption tests or the use of single factor serums. Phase 2 proved to be different from that organism since component 3 was lacking. The antigenic formula is VI₁, VIII: eh—1,2

ASSOCIATION OF THE VIRUS OF LYMPHOCYTIC CHORIOMENINGITIS WITH ERYTHROCYTES OF INFECTED ANIMALS

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The virus of lymphocytic choriomeningitis is found in organs and body fluids of infected man and animals [Rivers and Scott (1935, 1936), Armstrong, Wooley and Onstott (1936), Traub (1935, 1936a, c), Smadel and Wall (1941, 1942), Findlay and Stern (1936) and Kreis (1937)]. Traub (1936b) reported that the cellular sediment of leucocytes and erythrocytes from the blood of one out of four mice tested contained the virus.

In attempts to determine whether the mechanism of transfer of the virus through the blood bears any relation to the pathogenesis of the disease the following observations were made:

Erythrocytes from heparinized blood of infected mice and guinea pigs were separated from the silvery layer of leucocytes, washed 6-7 times in Locke solution

TABLE 1

STRAIN	ANIMAL SPECIES	VIRULENCE	INFECTIVITY OF ERYTHROCYTES
W. E.	guinea pig	high	consistent
W. E.	mouse	moderate	absent or irregular
F. A.	guinea pig	high	consistent
F. A.	mouse	moderate	absent
W. W. S.	guinea pig	low	absent or irregular
W. W. S.	mouse	high	consistent
T.	guinea pig	low	absent or irregular
T.	mouse	low	absent or irregular

and hemolyzed by addition of distilled water in proportion 1:3. The stroma of hemolyzed cells was also washed 6-7 times in Locke solution. The preparations each injected intracranially into several mice in a dose of 0.03 ml. were, as follows: blood serum; erythrocyte washings; the supernatant, washings of the sediment, and the sediment of hemolysed erythrocytes; and intact erythrocytes.

The virus was recovered from the erythrocytes of infected mice and guinea pigs. Depending on the virulence for the host, strains¹ of the virus differed from one another in their ability to enter into association with the erythrocytes of mice and guinea pigs. The results are summarized in table 1.

When a strain showed a decidedly greater virulence for one species than the other, there was observed consistent infectivity of erythrocytes of the species for which the strain possessed the greater virulence. The virus was recovered only irregularly from erythrocytes of mice and guinea pigs when the strain possessed low virulence for both species.

The concentration of the virus in the erythrocytes had no relationship to the

¹ The author is grateful to Drs. T. M. Rivers, J. E. Smadel and L. Thomas for supplying all the strains used in this investigation.

amount of the virus in the blood serum; the virus could be obtained from the hemolysed erythrocytes when the washings preceding hemolysis were free from the virus; although intact erythrocytes also proved infectious, the yield of the virus was lower than in hemolysed erythrocytes. Hemolysis failed, however to liberate the entire amount of virus associated with erythrocytes, since as many as 6-7 washings of the stroma, as well as the washed stroma itself remained infectious.

Thus, there occurs a firm association of the virus of lymphocytic choriomeningitis with the erythrocytes of infected mice and guinea pigs. The ability of the virus to enter into this association markedly depends on the virulence of the strain for the animal species infected. Consistent infectivity of the erythrocytes is observed when the strain is capable of eliciting in the animal species a severe and fatal infection.

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ANTIBACTERIAL ACTION OF A PYRIDINE ANALOGUE OF THIAMINE

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The pyridine analogue of thiamine, 2-methyl-4 amino-5-pyrimidyl-methyl-(2-methyl-3- β -hydroxyethyl) pyridinium bromide, has been reported by Robbins (1941) to be without thiamine activity for fungi and actually to inhibit their growth. Wooley and White (1943) have produced a thiamine deficiency syndrome in mice by feeding this analogue which they have named pyrithiamine. We have studied the quantitative aspects of the competition between pyrithiamine and thiamine as measured by bacterial growth and are reporting an

spirocheticidal action by the sera of a small series of rabbits infected for varying periods of time was observed. Sera with 33 Kahn units were no more spirocheticidal than others with no detectable amounts of reagin. In these experiments fresh sera, inactivated sera, and inactivated sera plus guinea pig complement were employed. Test portions were examined by dark field at stated intervals.

THE CLINICAL ASPECTS OF SYPHILIS. *R. M. Sorenson*, Kansas State Board of Health, Topeka.

FURTHER STUDIES OF THE EFFECT OF THE MEDIUM ON THE APPARENT SURVIVAL OF HEAT-TREATED BACTERIA. *F. E. Nelson*, Kansas State College.

Previous studies from this laboratory have shown that the apparent survival of heat-treated bacteria can be influenced markedly by the plating medium. The studies reported here have been obtained to show the effects of modifying a 0.5 per cent glucose, 0.1 per cent ammonium dihydrogen phosphate, 0.1 per cent potassium monohydrogen phosphate, 1.5 per cent agar basal medium by addition of complex nitrogenous substances. *Escherichia coli* and thermoduric lactic streptococci were used as the test organisms. The cultures were diluted in sterile skim milk to give counts of fifteen to twenty-five million per ml., and these suspensions were heated to the extent that the majority of the bacteria present failed to develop under the experimental conditions employed. The unheated suspensions were used as controls. The unsupplemented basal medium failed to support growth of the unheated control cultures of the thermoduric lactic bacteria but was adequate for unheated *E. coli*. All of the supplemented media gave uniform counts within series of the unheated control cultures.

A medium containing 0.05 per cent Tryptone added to the basal medium before autoclaving proved superior to similar media made with Bacto peptone, Neopeptone or skim milk for giving the maximum growth of heat-treated bacteria. As the amount of Tryptone was decreased from 0.5 to 0.01 per cent in the medium, the counts of heat-treated bacteria declined to the levels

characteristic of the basal medium to which 0.1 ml. of milk had been added with the bacterial suspension. When 0.01 per cent thioglycollic acid was added to the basal medium supplemented with 0.01 per cent Tryptone, this medium gave counts on suspensions of heat-treated bacteria roughly equal to the counts on basal medium supplemented with 0.2 per cent Tryptone without added thioglycollic acid.

When 0.5 per cent Tryptone was added to the basal medium before autoclaving, the resulting medium yielded much higher counts on heat-treated cultures than did a similar medium in which the Tryptone had been incorporated as a sterile 10 per cent solution immediately before the agar was poured into the plates.

The conclusion was reached that probably 0.01 per cent Tryptone is an adequate supplement to the basal medium for nutritional requirements of the organisms, but the larger quantities of Tryptone added prior to sterilization permit the poisoning of the medium at a potential sufficiently reducing to allow development of a greater proportion of the heat-treated bacteria.

THE OCCURRENCE OF NATURAL ANTIBODIES AGAINST CERTAIN YEAST-LIKE FUNGI. *Charles H. Drake*, Department of Bacteriology, University of Kansas.

The presence of antibodies against certain yeast-like fungi was shown by means of slide agglutination for 100 human and 50 rabbit sera. This technique avoids the difficulties from rapid sedimentation such as occurs with these organisms in tube agglutinations. 94 per cent of the human and 84 per cent of the rabbit sera showed agglutination for at least one of the organisms used. None of the sera showed agglutinins for *Rhodorula glutinosa*. The pattern of the results indicates that these are probably natural antibodies.

Candida albicans was agglutinated by 88 per cent of the human and 70 per cent of the rabbit sera. The titre was as high or higher than that for the other organisms in 70 per cent of the human and 18 per cent of the rabbit sera. *Candida kruzei* was agglutinated by 21 per cent of the human and 66 per cent of the rabbit sera with the titres as high or higher in 2 per cent of the human and 26 per cent of the rabbit sera. *Saccharomyces*

cerevisiae showed a reaction with 56 per cent of the human and 70 per cent of the rabbit sera with the titre as high or higher in 26 per cent of the human and 60 per cent of the rabbit sera. *Torulopsis pulcherrima* was agglutinated by 62 per cent of the human and 68 per cent of the rabbit sera with titres as high or higher in 30 per cent of the human and 18 per cent of the rabbit sera.

THE NEWTON EPIDEMIC. *Charles A. Hunter*
Flora Acton and Harle Barrett, Kansas'
State Board of Health.

An epidemic of approximately 3,000 cases of dysentery occurred in Newton, Kansas, in September, 1942. The organisms isolated and identified were *Shigella para-dysenteriae* Hiss, *Salmonella schottmulleri* and *Salmonella typhi-murium*. The *Salmonella* cultures were typed by means of antigenic analysis. The water supply was found to be contaminated due to cross connection with sewerage system. Two deaths occurred and upon autopsy the dysentery bacilli were isolated.

BACTERIOLOGY OF EGGS. V. D. Foltz,
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THE INHIBITORY EFFECT OF CHLORMERCURI
CARVACROL ON THE GROWTH OF PATHO-
GENIC FUNGI. *Carl E. Georgi*, Department
of Bacteriology, University of Nebraska.

Chlormercuri carvacrol, and combinations of this compound with benzoic and salicylic acids, were tested for their fungistatic and fungicidal activity against microorganisms associated with dermatophytosis commonly termed "athlete's foot."

Test organisms were five filamentous fungi,

Epidermophyton inguinale, *E. interdigitale*, *Trichophyton purpureum*, *T. gypseum* and *T. rosaceum*, two yeasts, *Monilia albicans* and *M. candida* and a bacterium *Staphylococcus aureus*. The pathogenic yeasts were studied because of their relation to blastomycoses and frequent association with multiple dermatophytoses while *S. aureus* was included because of its omnipresence on the surface of the skin.

All investigations on ointments were conducted "in vitro" employing a modification of the agar cup-plate technique in which the fungistatic action is measured by a zone of inhibition around the cup containing the test ointment. Fungicidal activity was estimated by means of a modified phenol coefficient test using *T. rosaceum* as the organism.

Chlormercuri carvacrol, in combination with salicylic and benzoic acids, was found to be considerably more inhibitory than any of the recognized standard preparations used for comparative studies.

Variations in the degree of resistance to fungistatic agents was found to vary with the species of fungus under investigation. *T. rosaceum* was consistently the most resistant.

The presence of 10 per cent blood serum reduced the fungistatic action of the test compounds.

When the fungistatic agents were incorporated in ointments made of "greasy bases," the zone of inhibition was less than when these same agents were made up in hydrophilic bases.

Sulfathiazole, reported to be effective in the treatment of "athlete's foot," was found to be without inhibitory activity under the conditions of the experiment.



Arthur Trautwein Henrici
1889-1943

Arthur Trautwein Henrici¹

1889-1943

With the death of Dr. Arthur T. Henrici, professor of bacteriology at the University of Minnesota, there passed away one of the most original and outstanding bacteriologists in America. His ingenuity, broad perspective, and creative ability won for him high regard in his chosen profession of microbiology; his personal charm endeared him to his many scientific colleagues. He will long be remembered as a profound scholar, a stimulating teacher, and a genial friend.

He was trained in medicine and served as a pathologist in his first appointment, but, subsequent to assuming duties at the University of Minnesota, became interested in the broader aspects of bacteriology, more accurately designated by the term microbiology. His studies took him outside the field of medical bacteriology into bacterial morphology, taxonomy, ecology, and physiology; he went considerably beyond the limits of bacteriology itself to embrace such related groups of microorganisms as the actinomycetes and fungi. These organisms, to which he sometimes referred as "higher bacteria," were subjects of studies in morphology and taxonomy and he was an authority on their relationships to human diseases. He enriched the field of microbiology through investigations in such diverse subjects as pathology, bacterial morphology, and limnology.

A clear thinker, Dr. Henrici envisaged microorganisms as a biological group independent of either plants or animals, in spite of their similarities to these organisms and their development in association with them. This concept dominated his "Biology of Bacteria" and did much to stimulate interest in microorganisms, irrespective of their relations to disease, their development in soil and in food, or their industrial significance. He was both a keen observer and an able interpreter of the biology of microorganisms.

Born in 1889 (March 31) in Economy (now Ambridge), Pennsylvania, Dr. Henrici attended public school in Pittsburgh and received his medical training at the University of Pittsburgh, from which he was graduated in 1911. After a year and a half as pathologist at St. Francis Hospital, he was called in 1913 to the University of Minnesota, where first as Instructor, then as professor, he spent his remaining life, with the exception of about two years in France during World War I as a captain in the Medical Corps.

He married Blanche Ressler in 1913 and had three children, a son Carl Ressler and two daughters Ruth Elizabeth and Hazel Jean. He was a kind father and took great pride and joy in his family.

Among the many contributions of Dr. Henrici to microbiology, his studies on morphologic variation and the rates of growth of bacteria deserve particular attention. He elucidated in a clear and concise manner that order can be "brought out of the chaos which has so far filled that field of bacteriology which

¹ President, Society of American Bacteriologists, 1939.

has to deal with the form and structure of bacterial cells." He demonstrated that "contrary to the orthodox teaching, the cells of bacteria are constantly changing in size and form and structure, but that instead of these changes occurring in a haphazard or meaningless fashion, or instead of being phases in a rather vague and complex life cycle, they occur with great regularity and are governed by relatively simple laws which, after more data have been accumulated and analyzed, may probably be very precisely formulated." He contributed to a better understanding of the characteristics of "Molds, Yeasts and Actinomycetes," and of their participation with bacteria in transformations in nature. Through the medium of his widely accepted text on "The Biology of Bacteria," he enlivened the interest of many a young student in bacteria. His lucid and fascinating presentation had a freshness which is seldom encountered in a textbook. Among his notable contributions it is sufficient to mention his work on the ecology of fresh-water bacteria and later of salt-water bacteria, on their nature and distribution, as well as their role in the cycle of life in the lake and sea.

He was broadminded and liberal; this expressed itself in his social outlook as well as in his scientific activities. By nature, he was an artist and he developed hobbies in etching, painting, and photography to which he devoted himself with characteristic enthusiasm and in which he attained marked excellence. He prepared illustrations for most of his publications and by these he elucidated changes in bacterial morphology with much greater accuracy than written words could express.

Soft-spoken, sympathetic, modest and artistic, he commanded the confidence and admiration of his associates, colleagues, and students alike. His passing is a great loss to the science of bacteriology, which he made his own and which he enriched through both his published contributions and his personal influence.

ROBERT L. STARKEY
SELMAN A. WAKSMAN

SOME CHARACTERISTICS OF GREEN-FLUORESCENT PIGMENT-PRODUCING BACTERIA

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Since Schroeter (1872) first isolated and described an organism producing a green-fluorescent pigment, information on this particular type of organism has consistently appeared in the literature. Růžička (1899) studied a small group of fluorescent organisms and concluded that the group could be separated into two parts, one closely related to the semi-pathogenic *Bacterium pyocyaneum*, the other to *Bacterium fluorescens-liquefaciens*. A study of 15 organisms convinced Niederkorn (1900) that there were only two constant species. Jordan (1903) separated 58 strains into two groups on the basis of gelatin liquefaction. Edson and Carpenter (1912) separated 42 strains, isolated from Vermont maple sap, into seven groups, on the basis of hydrogen sulphide production, reduction of nitrates, growth on Uschinsky's solution, growth on Cohn's nutrient solution and action on gelatin and milk. They concluded that representatives of the seven groups plus six other species can not be sufficiently well differentiated to warrant calling them other than "liquefaciens" or "non-liquefaciens" strains of *Pseudomonas fluorescens*. Tanner (1918) found in the literature more than 95 species, many of which were incompletely described. He placed 100 strains which he isolated from water into 27 groups. However, he suggests that gelatin liquefaction and nitrate reduction are sufficiently variable to limit his groups to nine. Sherwood, Johnston and Radotinsky (1926) presented incomplete data on 22 strains of *Bacillus pyocyaneus*, giving data on carbohydrate reactions, hydrogen sulphide, indole, and hydrogen cyanide production, and nitrate reduction. Monias (1928) divided 30 strains into five groups; however, only three of his groups produced pigment. These three groups were formed on the basis of morphology and carbohydrate fermentations. Reid, Harris, Naghski and Gatchell (1941) studied 600 *Pseudomonas* cultures and found in addition to serological differences two types of flagellation and a difference in the ability to grow at 37°C.

Early investigations indicated that pigmentation was inconsistent. Gessard (1890) reported that various cultural methods caused *Bacillus pyocyaneus* to form the fluorescent pigment alone, pyocyanin alone or both pigments together. Charrin and Phisalix (1892) recorded the failure of pigment production by growing *Bacillus pyocyaneus* at 42.5°C. Jordan (1899) suggested: "Except for the occasional loss of one or another function, the different varieties are not so plastic as sometimes assumed and can not be readily converted into one or another by subjection to varying conditions of life." Eisenburg (1914) found that seven stock cultures varied in pyocyanin and fluorescein production. Bac-
Bac-

lein (1918) obtained variants containing no pigment, the fluorescent pigment alone, or both pigments, from a single culture.

Because of the variability in pigment production by *Pseudomonas* cultures, more than the usual amount of work has been devoted to the development of a medium upon which the production of fluorescence is reproducible. Neelsen (1880), working with impure cultures from blue milk, proposed a synthetic medium containing ammonium tartrate, neutral potassium phosphate, magnesium sulphate and calcium chloride. Gessard (1892) using a medium similar to Neelsen's, found phosphates essential. Thumm (1895) reported that, in addition to an organic source of nitrogen, phosphates and magnesium sulfate must be present for fluorescence to occur. Georgia and Poe (1931) in careful work reaffirmed the necessity of magnesium, phosphate and sulfate, showing them to be essential for the production of fluorescence. Possibly the alteration of the chromogenic function may be caused also by inhibitory substances in the medium. Fuller and Johnson (1899) stated that heavy metals prevent pigmentation. Turfitt (1936) stated that the minute amounts of heavy metal salts found in tap water are sufficient to inhibit all chromogenesis. Kharasch, Conway, and Bloom (1936) have shown that one part of copper in 50,000 will cause inhibition of pigment production. They also list several organic compounds preventing pigmentation. Turfitt (1936), prompted by the constancy of pigmentation on the appropriate medium, used the chromogenic function as a basis for the classification of the genus *Pseudomonas*. Turfitt (1937) found that concentrates of the fluorescent pigment from *Bacillus pyocyaneus*, *Bacillus fluorescens-liquesfaciens* and *Bacillus fluorescens-non-liquesfaciens* had the same chemical properties.

Since knowledge of the green-fluorescent organisms is limited and often contradictory, a comparative study of a representative number of this type of bacteria seemed desirable.

METHODS AND RESULTS

In addition to 30 stock cultures, 169 green-fluorescent organisms were obtained by isolations from milk, butter, lactose, milk bottles, water, ground meat, fish, rabbit sera, bone meal, lettuce, wood pulp, soil, manure, and sewage.

Morphologically, this study concerned itself chiefly with the form of the bacterium, its gram reaction, and the position of flagella. All these organisms are gram-negative rods which occur singly, in pairs, or in very short chains. With the exception of one culture, all are motile, bearing one to several flagella in a tuft at one end of the cell. The exception was a dissociant of culture 25. Colonies on nutrient agar plates were surrounded by the water-soluble pigment but otherwise had no very distinguishing characteristics which would easily set them apart from other members within the group.

Fluorescent pigment was consistently produced by all these organisms when grown on Georgia and Poe's asparagine, dipotassium phosphate, magnesium sulphate medium. Seventy-one of 199 cultures failed to produce visible pigment on 0.5 per cent tryptone (Difco) containing the necessary inorganic constituents

even when the broth was made from water redistilled from glass. Sodium lactate, when used as the sole source of carbon, was the only organic acid salt tested which produced a degree of fluorescence comparable to that produced on the asparagine medium. However, 21 cultures failed to produce fluorescence on this medium.

Our findings show that pyocyanin-producing cultures always produced pyocyanin on a medium consisting of 5 per cent glycerol, 2 per cent peptone, and 3 per cent agar (Gessard, 1891). On this medium fluorescence is inhibited. No better pyocyanin production nor additional pyocyanin-producing cultures were detected by growing on nutrient agar, glucose agar, tryptone broth, tryptone cyanide broth or nutrient broth. The pyocyanin-producing cultures grew on Georgia and Poe's synthetic medium. On this medium fluorescent pigment was produced, but little or no pyocyanin was observed.

In pigment-production studies, the cultures were incubated at room temperature for one week; observations were also made at the end of three days. Our findings, as well as observations of many other workers on this group, indicate that pyocyanin tends to be produced more quickly and more abundantly at 37°C., whereas the green-fluorescent pigment is more readily observed at temperatures ranging from 20°C. to 30°C. In order to observe with ease fluorescent pigment production, it is only necessary to grow the cultures in question on Georgia and Poe's asparagine medium for one week at room temperature. If some of the pigment-producing cultures also produce pyocyanin, this can be readily observed by growing the cultures at 37°C. on Gessard's glycerol agar.

Proteolysis and change in pH were determined on litmus milk, the cultures being incubated at room temperature for three weeks. Three types of reaction were plainly visible. In one type of reaction, the milk casein was at least 75 per cent dissolved and frequently completely liquefied at the end of the incubation period. In another type of reaction, there was a very obvious increase in alkalinity of the milk with no visible proteolytic action or curd formation. In a third type of reaction, a few cultures left the milk unchanged.

The results reported in Table I show the first six groups to digest the casein of milk, the next six groups to cause the milk to become basic without visible digestion of the casein, and the last two groups to produce no observable change in milk.

Inoculated tubes of beef-extract peptone 4 per cent gelatin were held at room temperature for three weeks to test the ability of the cultures to liquefy gelatin. Liquefaction was determined by holding in ice water inoculated and incubated tubes along with uninoculated controls. More than 70 per cent of the organisms studied by us liquefied gelatin. All the cultures in the first six groups which digested milk also liquefied gelatin. The seven cultures of group VII liquefied gelatin and caused the milk to become basic, but gave no visible evidence of casein digestion. The remaining cultures failed to liquefy gelatin.

When grown in 1 per cent sodium nitrate in beef-extract peptone broth, in tubes containing inverted vials, for seven days at room temperature, approximately 39 per cent of the cultures reduced nitrates to gaseous nitrogen or nitrogen

compounds; 9 per cent reduced nitrates to nitrites, and more than half had, by this method, no detectable action on nitrates.

From cultures grown in 0.1 per cent tryptone, inoculations were made into a medium consisting of 0.5 per cent dipotassium phosphate, 0.5 per cent sodium nitrate, 0.05 per cent magnesium sulphate and 0.5 per cent glucose (pH 6.8–7.0). After five days, transfers were made into a second tube of the same composition. Growth in this medium was detected by turbidity. All but 7.5 per cent of the cultures grew in this medium, indicating their ability to utilize nitrates as their only source of nitrogen. Nitrites were detected in 86 per cent of the cultures. Although gas production in the synthetic medium was more difficult to detect, all cultures producing gas on the usual medium also produced gas on the synthetic medium. Table I shows that cultures of Groups I, II, and VIII produce gaseous forms of nitrogen from nitrates.

By substituting sodium nitrite for sodium nitrate as the only nitrogen source in the synthetic medium, it was found that no culture initiated growth in 0.4, 0.1, 0.05, or 0.01 per cent sodium nitrite. In the green-fluorescent, pigment-producing group, nitrites do not function as a nitrogen source. When some other nitrogen source, such as nitrates or tryptone is added to the nitrite solutions, growth occurs and nitrites may be reduced.

When grown in a 1 per cent tryptone solution, none of the organisms produced indole. Some pyocyanin-producing cultures produced a faint pink color 15–30 minutes after adding the reagents. Although this color conceivably might be mistaken for a faint indole test, it is actually the acid color of pyocyanin which is an acid-base indicator.

In their ability to produce ammonia when cultured in peptone broth tubes, the organisms differed only quantitatively. All of the cultures produced ammonia.

In the ordinary nutrient broth to which a carbohydrate had been added, the proteolytic action of these *Pseudomonas* organisms disguised their fermentative ability. Therefore, a different nitrogen source was substituted by using a medium consisting of 0.5 per cent ammonium sulphate, 0.5 per cent dipotassium phosphate, and 1 per cent of the carbohydrate to be tested. On this synthetic medium, cultures unable to ferment the carbohydrate failed to grow. All cultures produced acid from glucose and glycerol; 19 per cent of the cultures fermented sucrose; none attacked lactose. A medium containing 0.1 per cent tryptone and 1 per cent of the carbohydrate tested gave the same fermentative reactions as the synthetic medium. When grown on streaked starch agar plates, none of the organisms tested by us was able to hydrolize starch.

Utilization of organic acid salts was demonstrated by the growth of cultures on a synthetic medium consisting of 0.5 per cent dipotassium phosphate, 0.5 per cent ammonium sulfate, 0.05 per cent magnesium sulphate, and 0.3 per cent of the carbon source (pH 6.8–7.0). All of the cultures tested were able to utilize citrates; no organisms utilized formates; acetates, lactates, and tartrates were utilized by some strains.

The breakdown of fat, a character frequently attributed to this group, was also

TABLE I

Some possibly significant biochemical reactions of Pseudomonas organisms

GROUP	NO. OF CULTURES	GROWTH AT		ACTION ON MILK	LIQUEFACTION OF GELATIN	REDUCTION OF NITRATES	PRODUCTION OF PYOCYANIN	UTILIZATION OF			
		5°C.	42°C.					Sucrose	Acetic acid	Lactic acid	Tartaric acid
I a	37	—	+	P	+	(+)	+	—	+	+	—
b	4	—	+	P	+	(+)	—	—	+	+	—
II a	20	+	—	P	+	(+)	—	—	+	+	—
b	1	+	—	P	+	(+)	—	+	+	+	—
c	6	+	—	P	+	(+)	—	—	—	+	—
d	3	+	—	P	+	(+)	—	—	+	+	+
III a	9	+	—	P	+	+	—	—	+	+	—
b	3	+	—	P	+	+	—	—	—	+	—
c	1	+	—	P	+	+	—	+	+	+	—
d	2	+	—	P	+	+	—	+	—	+	—
IV a	16	+	—	P	+	—	—	+	+	+	—
b	1	+	—	P	+	—	—	+	+	+	+
c	3	+	—	P	+	—	—	+	—	—	—
d	16	+	—	P	+	—	—	—	+	+	—
e	4	+	—	P	+	—	—	—	+	+	+
f	2	+	—	P	+	—	—	—	+	—	—
g	1	+	—	P	+	—	—	—	—	+	—
h	1	+	—	P	+	—	—	—	—	—	—
V	2	—	—	P	+	—	—	—	+	+	—
VI	1	+	—	P	+	—	—	—	+	+	—
VII	7	+	—	B	+	—	—	+	—	—	—
VIII a	4	+	—	B	—	(+)	—	—	+	+	—
b	1	+	—	B	—	(+)	—	+	+	+	+
c	1	+	—	B	—	(+)	—	+	—	+	+
IX a	1	+	—	B	—	+	—	—	+	+	+
b	1	+	—	B	—	+	—	—	+	+	—
X a	25	+	—	B	—	—	—	—	+	+	—
b	15	+	—	B	—	—	—	—	+	+	+
c	1	+	—	B	—	—	—	—	—	+	—
d	3	+	—	B	—	—	—	+	+	+	—
e	1	+	—	B	—	—	—	—	+	—	—
XI a	1	—	—	B	—	—	—	—	+	+	—
b	1	—	—	B	—	—	—	+	—	—	—
c	1	—	—	B	—	—	—	—	—	+	+
XII	1	+	+	B	—	—	—	—	+	+	+
XIII	1	+	—	—	—	—	—	—	+	+	—
XIV	1	—	—	—	—	—	—	—	—	+	—

P = Digestion of the casein of milk. B = Basic to litmus. (+) = Reduction of nitrates to nitrites and gas.

tested. The fat to be used in these tests was prepared in the following manner. Salted butter of good quality was melted at 45°C. The fat was separated from the gross protein present by decanting. Further separation of fat from its impurities was accomplished by washing in a warm 50-per-cent alcohol solution. The liquid fat and the alcohol were allowed to separate by gravity and the alcohol was drawn off with the aid of a separatory funnel. After the third washing, the liquid fat layer was centrifuged for 15–30 minutes. The fat was pipetted off and filtered through a Seitz filter. One-half ml. portions of the filtrate were pipetted off into tubes containing approximately 9–10 ml. of a sterile medium consisting of 0.5 per cent dipotassium phosphate, 0.5 per cent ammonium sulfate, and 0.05 per cent magnesium sulfate (adjusted to pH 6.8–6.9). Tubes containing this medium were inoculated with one drop of a 24-hour culture in a 0.1 per cent tryptone broth. It is not possible to state positively that the preparation used in these tests contained no impurities. Two methods were employed to determine the ability of these organisms to act upon the fat. One was growth, as indicated by turbidity in the medium, the assumption being that growth could not occur without utilization of the fat preparation as a source of carbon. The second method was to observe the physical change and breakdown occurring in the fat layer. Those observations are recorded in Table II. Although, in many cases, turbidity was very slight, 86 per cent of the fluorescent organisms exhibited a detectable growth. Only 25 per cent, however, attacked fat strongly enough to make it apparent from macroscopic observations. Sixty per cent of this definitely lipolytic group were found to be of the high temperature *Pseudomonas aeruginosa* type. All but three organisms, found to be lipolytic, were also proteolytic.

In studies on the streptococci, mycobacteria, and bacilli, the temperature limits of growth are known to be very significant. Similar studies have been made on these *Pseudomonas* cultures, the results of which are also believed to be significant. By this means, all but seven cultures studied fit into one of two groups: one group, 21 per cent of the cultures studied, grew at 42°C. and failed to grow at 5°C.; another group, 76 per cent of the cultures studied, grew at 5°C. but failed to grow at 42°C. These results are recorded in Table I, where other correlating characteristics may be observed.

None of these *Pseudomonas* cultures was able to resist heating at 60°C. for 30 minutes.

Resistance to phenol or cyanide was determined by growth in 0.3 per cent meat extract, 0.5 per cent tryptone broth containing varying amounts of phenol or cyanide. All cultures but one grew in 0.05 per cent phenol; none grew in 0.2 per cent phenol. All but 10 cultures grew in 0.05 per cent cyanide; none grew in 0.2 per cent cyanide. The pyocyanin-producing cultures tended to be more resistant to phenol and cyanide than were non-pyocyanin-producing cultures, although the correlation was imperfect. These variations may be noted in Table II.

The inhibition of growth of these organisms by varying concentrations of sodium chloride was determined using Georgia and Poe's asparagine synthetic

TABLE II
Some additional biochemical reactions of Pseudomonas organisms

GROUP	NO. OF CULTURES	GROWTH IN PHENOL				GROWTH IN NaCN				GROWTH IN NaCl				ACTION ON FAT		ACTION ON NITRATES IN A SYNTHETIC MEDIUM	
		.05%	.10%	.15%	.2%	.05%	.10%	.15%	.2%	3%	4%	5%	6%	Growth	Hydrolysis	Growth	Reduction
I a	37	+	+	33+	-	+	+	17+	-	+	36+	-	-	34+	29+	+	(+)
b	4	+	+	3+	-	+	+	-	-	+	+	-	-	+	2+	+	(+)
II a	20	+	18-	-	-	+	15-	-	-	+	18+	-	-	18+	1+	+	(+)
b	1	+	-	-	-	+	+	-	-	+	+	-	-	+	-	+	(+)
c	6	+	5-	-	-	+	-	-	-	+	+	-	-	4+	1+	+	(+)
d	3	+	-	-	-	+	-	-	-	+	+	-	-	2+	2+	+	(+)
III a	9	+	5+	-	-	+	6+	-	-	8+	7+	-	-	6+	1+	+	+
b	3	+	2-	-	-	+	2-	-	-	+	-	-	-	+	-	+	+
c	1	+	-	-	-	+	-	-	-	+	+	-	-	+	1+	+	+
d	2	+	-	-	-	+	-	-	-	+	1+	-	-	1+	-	+	+
IV a	16	+	10-	-	-	+	14-	-	-	+	14+	-	-	15+	3+	+	15+
b	1	+	+	-	-	+	-	-	-	+	+	-	-	+	-	+	-
c	3	+	-	-	-	+	-	-	-	2+	-	-	-	+	-	+	1+
d	16	+	12-	-	-	+	9+	15-	-	+	13+	-	-	15+	6+	+	12+
e	4	+	3-	-	-	+	2-	-	-	+	+	-	-	+	3+	+	2+
f	2	1+	-	-	-	+	1-	-	-	+	+	-	-	+	-	+	+
g	1	+	-	-	-	-	-	-	-	+	+	-	-	-	0	+	+
h	1	+	-	-	-	+	+	-	-	+	+	-	-	+	-	+	+
V	2	+	+	-	-	+	+	1+	-	+	-	-	-	+	1+	+	+
VI	1	+	+	-	-	+	-	-	-	+	-	-	-	+	-	+	-
VII	7	+	6-	6-	-	6-	-	-	-	4-	-	-	-	+	2+	6+	4+
VIII a	4	+	-	-	-	+	-	-	-	+	3+	-	-	+	-	+	(+)
b	1	+	-	-	-	+	-	-	-	+	-	-	-	+	-	+	(+)
c	1	+	-	-	-	+	-	-	-	+	-	-	-	+	-	+	(+)
IX a	1	+	+	-	-	+	-	-	-	+	-	-	-	+	-	+	+
b	1	+	-	-	-	+	-	-	-	+	-	-	-	+	-	+	+
X a	25	+	13+	24-	-	24+	15-	23-	-	+	20+	24-	-	22+	1+	24+	21+
b	15	+	14+	-	-	14+	9-	13-	-	+	13+	12-	-	11+	2+	+	+
c	1	+	-	-	-	+	-	-	-	+	-	-	-	+	-	+	+
d	3	+	2+	-	-	2+	2+	-	-	+	+	2+	-	+	-	+	+
e	1	+	-	-	-	+	-	-	-	+	-	-	-	-	0	+	+
XI a	1	+	+	-	-	+	+	-	-	+	+	-	-	+	-	+	+
b	1	+	-	-	-	+	-	-	-	+	-	-	-	-	0	+	+
c	1	+	-	-	-	+	-	-	-	+	-	-	-	+	-	+	-
XII	1	+	+	-	-	+	-	-	-	+	+	+	-	+	-	+	+
XIII	1	+	+	-	-	+	-	-	-	+	-	-	-	+	-	+	-
XIV	1	+	+	-	-	+	-	-	-	+	-	-	-	-	0	+	-

medium. Practically all cultures were able to grow in the presence of 3 per cent sodium chloride; very few grew in 5 per cent; and none grew in 6 per cent sodium chloride. These findings are recorded in Table II.

Conflicting reports are available concerning the aerobiosis of *Pseudomonas* organisms. Our findings convince us that they are obligate aerobes. Meat-infusion agar-shake cultures indicated that the organisms were obligate aerobes. Cultures inoculated into Georgia and Poe's synthetic medium were subjected to a week's incubation at room temperature under anaerobic conditions produced by a modification of Rosenthal's chromium sulfuric acid method for obtaining anaerobiosis (Mueller and Miller, 1941). Under these conditions, none of the *Pseudomonas* cultures tested by us were able to initiate growth.

DISCUSSION OF RESULTS

Polar flagella, gram-negative staining, elaboration of a fluorescent pigment, growth at 20°C. and 30°C., obligate aerobic relation to oxygen, failure to form indole, production of ammonia, and a weak attack on carbohydrates indicate very strongly that the organisms studied are very closely related. When grown on a buffered synthetic medium, all the cultures tested acted upon glucose, glycerol and citric acid, failed to attack lactose and formic acid, and were unable to produce indole. That this homogeneity does not extend into every physiological character is apparent from Tables I and II. Table I includes all the tests by which the organisms may be differentiated into groups and subgroups. Tests, correlating less with other characters or showing but slight relationship to other characters, are recorded in Table II.

The 41 organisms of Group I are separated from the rest by their ability to grow at 42°C. and failure to grow at 5°C. That this separation is justified is borne out by the fact that all 41 organisms are proteolytic on milk and gelatin, reduce nitrate to gaseous nitrogen compounds, attack glucose but not lactose or sucrose, utilize glycerol and the same organic acids, and appear to have a higher tolerance for phenol and sodium cyanide and a strong lytic action on fats. Both Group Ia and Group Ib have the cultural and biochemical properties of *Pseudomonas aeruginosa* (Schroeter) Migula, listed in Bergey's Manual of Determinative Bacteriology. Although Group Ib produced a fluorescent pigment, at no time did it produce pyocyanin. From the close correlation of its other physiological properties, we suspect that this group is a variant of Group Ia which has lost its pyocyanogenic power.

The organisms included in the remaining 13 groups all failed to produce pyocyanin and with the exception of seven cultures all failed to grow at 42°C. and grew at 5°C. The seven cultures, mentioned above, constitute Groups V, XI, XII, and XIV. The subgroups indicated by letters in Groups II to XIV are made on the basis of their ability to utilize, as the sole source of carbon, sucrose, acetic acid, lactic acid, or tartaric acid. It is not contended that these subgroups are significant or justified; they are merely reported. The principal difference between Groups II and III is the inability of the cultures in Group III to reduce nitrates to gaseous nitrogen. Obviously, the reports of action on nitrates in Tables I and II require some explanation. Results (Table I) on the non-

synthetic medium were regularly reproducible; previous workers have used this medium; and the classification appearing in Bergey's Manual is based on this medium. Results with the synthetic medium were not as easily reproducible and have not been used before. On the basis of data here reported, it is our belief that the reduction of nitrates to N_2 or gaseous nitrogen compounds by cultures of the *Pseudomonas* group is a significant and easily distinguishable characteristic, but that the reduction of nitrates to nitrites is a character of minor distinguishing value.

Groups III, IV, V, and VI failed to produce gas from nitrates. The separation of one from the other is slightly arbitrary. Group V and also Groups XI and XIV were unable to grow at either 5°C. or 42°C. Group V, being strongly proteolytic, is believed to be distinctly different from Groups XI and XIV. Group VI, which resembled *Pseudomonas fragi* (Hussong, Long, and Hammer, 1937), was given separate status on the basis of its rapid reduction of litmus milk and the appearance of a narrow pink zone on the surface of litmus milk. When air was incorporated in the milk by shaking, the reaction was basic. The casein was partially digested.

The cultures in Group VII liquefy gelatin but fail to digest milk. All the cultures in this group were isolated from plants. Group VIII is distinguished by the ability of the cultures in it to produce gaseous nitrogen compounds from nitrates and resembles somewhat Bergey's description of *Pseudomonas denitrificans*. There is some similarity in the cultures of Group IX to Bergey's description of *Pseudomonas putida*. Some resemblance exists between the cultures in Group X and Bergey's description of *Pseudomonas ovalis* and *Pseudomonas convexa*. Groups IX, X, XI and XII are in our opinion only slightly different, their action on milk and gelatin being the same and their action on nitrates not being significantly different. The one culture constituting Group XII grew at both 5°C. and 42°C. The two cultures constituting Groups XIII and XIV produced no change in milk.

SUMMARY

A study of 199 green-fluorescent bacteria is reported. By appropriate culture, several characteristics were found to be common to all cultures. These were production of green-fluorescent pigment, rod form, polar flagella, gram-negative staining, obligate aerobic relation to oxygen, failure to form indole, production of ammonia, growth at 20° and 30°C., death at 60°C. for 30 minutes, and a weak attack on carbohydrates.

The cultures were divided into 14 groups on the basis of ability to grow at 5°C. and 42°C., action on milk, liquefaction of gelatin, and reduction of nitrates. Subgroups were established by the ability of the cultures to produce pyocyanin and to utilize sucrose, acetic acid, lactic acid, or tartaric acid as the sole carbon source.

Growth of the cultures in different concentrations of phenol, sodium cyanide and sodium chloride are reported, along with growth in and hydrolysis of fat and action on nitrates when grown in a synthetic medium.

The uniformity of the reduction of nitrates to nitrogen or gaseous nitrogen compounds and discrepancies in the reduction to nitrites are pointed out.

Where possible, names of species most nearly describing the groups in this report are used. Group I (*Pseudomonas aeruginosa*) is best defined. For accurate definition, limiting temperatures of growth must be used.

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A SIMPLE TECHNIQUE FOR THE DETECTION OF MELIBIOSE-FERMENTING YEASTS¹

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The fermentation of melibiose is given special importance in Stelling-Dekker's (1931) classification of the genus *Saccharomyces*. Two important groups of species are differentiated by their ability or inability to ferment this sugar. *Saccharomyces carlsbergensis* and *S. cerevisiae*, the two species which include the strains most commonly used in industrial processes, may be separated in this manner; the former ferments melibiose whereas the latter does not.

Melibiose itself is seldom used in determining the ability of a strain to ferment this sugar. Raffinose, a trisaccharide which is readily attacked by almost all of the industrially important yeasts, is used instead, for on partial hydrolysis it yields melibiose and fructose. The advantages in the use of raffinose rather than melibiose are threefold: first, raffinose costs about one-fourth as much as melibiose; second, raffinose provides twice as much information as does melibiose for it gives information regarding the yeast's ability to attack both of these sugars; and third, as will be shown presently, some strains attack melibiose more rapidly when it is liberated from the raffinose molecule than when it is supplied in the form of pure melibiose.

Several techniques have been devised for the raffinose-melibiose fermentation. The Dutch school uses a quantitative apparatus as described by Van der Haar (1920) for determining the amount of gas produced from a known amount of raffinose by the organism under test. If the organism ferments melibiose, it will produce much more gas than if it attacks only the fructose resulting from the partial hydrolysis of the raffinose.

At the New York Agricultural Experiment Station at Geneva (Manual, 1936) the Eldredge tube is used. This consists of two tubes connected by a horizontal arm to allow interchange of gas. The raffinose solution and yeast are placed in one tube and a definite quantity of barium hydroxide in the other. During incubation, the carbon dioxide evolved reacts with the barium hydroxide to form barium carbonate. After the fermentation is over, the excess hydroxide is titrated with a standard acid solution. Again, the strains which ferment raffinose completely will cause the formation of much more barium carbonate than do the strains which cannot attack melibiose.

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² The Northern Regional Research Laboratory is one of four regional laboratories authorized by Congress in the Agricultural Adjustment Act of 1938 for the purpose of conducting research to develop new uses and outlets for agricultural commodities. These laboratories are administered by the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

Zimmermann (1938) inoculated a raffinose tube with the yeast under test and after 14 days determined the amount of sugar remaining in the medium.

Henrici (1941) used melibiose rather than raffinose. The fermentation took place in a conventional fermentation tube.

Due to the limitations of some of these techniques and the special equipment required in others, a new simple technique based on raffinose fermentation has been devised. Basically, it consisted of inoculating a tube of 4 per cent raffinose broth, containing an insert, with the yeast under test. The basal medium contained 0.45 per cent Difco powdered yeast extract, 0.75 per cent peptone, and sufficient bromthymolblue to give a definite green color when adjusted to pH 7.0. Two milliliter quantities of the basal medium were placed in test tubes measuring 150 x 12 mm. outside diameter, containing inserts measuring 50 x 6 mm. After sterilization, 1 ml. quantities of sterile aqueous 12 per cent raffinose solution were added to the tubes. The raffinose solution was sterilized separately at 12 pounds steam pressure for 20 minutes. Inoculation was made from a young culture growing on a yeast-extract agar slant. Incubation was at 30°C. The amount of gas trapped in the insert was recorded at regular intervals of time. When the fermentation had passed its maximum and the volume of gas in the insert had begun to decrease, the tube was inoculated with young cells of *Saccharomyces carlsbergensis*, NRRL strain No. 379, a strong melibiose-fermenting yeast. If additional gas was produced following this second inoculation, the yeast under test had failed to ferment melibiose. On the other hand, if no gas was produced, this indicated that the yeast under test had already fermented the melibiose. Figure 1 illustrates the action of a melibiose-positive strain, NRRL No. 671, and a melibiose-negative strain, NRRL No. 631. The former shows no evolution of gas following the inoculation with strain No. 379, whereas the latter does.

The technique thus outlined was used in a survey of 200 strains of industrial yeasts included in the collection of the Northern Regional Research Laboratory. Seventeen strains were found which fermented both raffinose and melibiose. Nearly all of these 17 strains produced a simultaneous fermentation of these two sugars. That is, the gas readings showed a single peak of maximum gas production. The average period of time required to complete the fermentation and cause a decrease in the amount of gas in the inserts was 7 days, whereas the longest period was 16 days.

Four-per cent melibiose broth was inoculated with each of the 17 strains to check the preceding results. Most of the strains caused a fermentation of the melibiose within a few days following inoculation. However, one strain, No. 804, required 12 days to start a fermentation. Another, No. 636, produced no gas until 16 days after inoculation, although by the seventeenth day the insert was completely filled. A third strain, No. 236, required 14 days in one trial and 28 days in another before it began to ferment the pure melibiose (fig. 2). The fourth strain, No. 562, produced an alkaline reaction in the tube which evidently prevented the initiation of fermentation, for none took place throughout a period of 28 days. This was the only melibiose-positive strain, as judged by the raffinose test, which failed to ferment pure melibiose.

In view of the difficulty which some strains experienced in attacking pure melibiose, it was thought desirable to change the technique where raffinose is used as the source of melibiose. Instead of inoculating with the known melibiose fermenter as soon as the fermentation caused by the organism under test had subsided, this inoculation was postponed until a total incubation period of

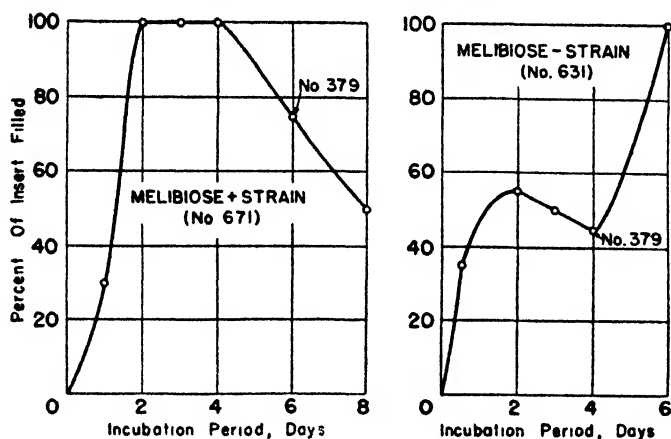


FIG. 1. ACTION OF TYPICAL MELIBIOSE-POSITIVE AND MELIBIOSE-NEGATIVE STRAINS OF YEASTS ON RAFFINOSE

The arrow denotes the point at which the fermentation medium was inoculated with the melibiose-positive strain No. 379.

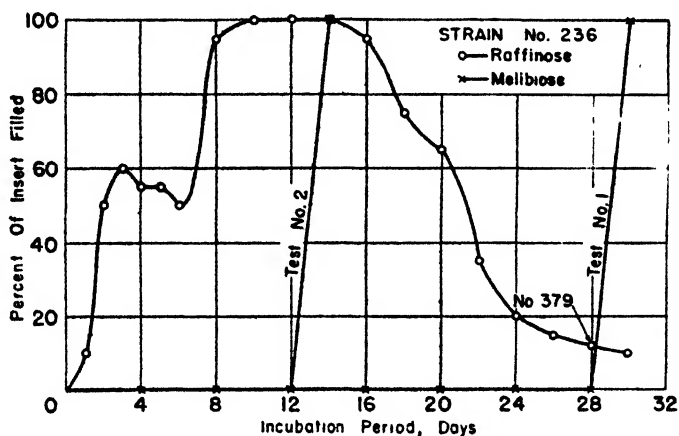


FIG. 2. COMPARATIVE FERMENTATION BY STRAIN NO. 236 OF MELIBIOSE IN THE PURE FORM AND WHEN LIBERATED FROM RAFFINOSE

Note the prolonged delay preceding the initiation of gas production in presence of pure melibiose.

28 days or more had elapsed. Fifteen days after inoculation, 1 ml. of sterile water was added to each tube to replace that lost by evaporation.

Eighty-five strains were run by this modified technique. Of this number, four strains were found to give a belated fermentation of melibiose. Three of these produced distinct and well separated raffinose and melibiose fermentations.

In two of these strains the minimum time required to start the melibiose fermentation was 18 days, while in the third strain it was initiated in 8 days. Figure 3, showing the behavior of strain No. 830, represents the type of action shown by these three strains on raffinose. As is illustrated, the attack on the liberated melibiose was relatively slow; nevertheless, when cells from this fermenting solution were transferred to a tube of pure melibiose broth, a very rapid fermentation was obtained.

The fourth yeast, strain No. 264, was in a class by itself. When grown in the 4 per cent raffinose medium for 50 days it had not utilized all of the sugars present. Yet it did produce gas from melibiose. It is not known whether this strain actually should be considered as a fermentative yeast, or whether it should be considered as having produced carbon dioxide by aerobic respiration. It always produced abundant growth throughout the tube and a marked pellicle

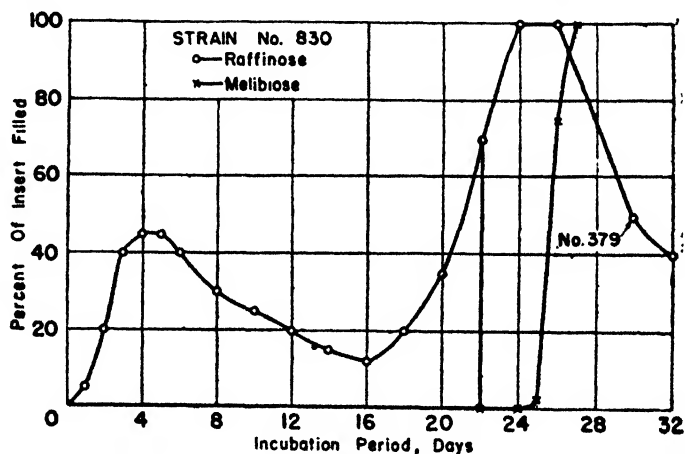


FIG. 3. DELAYED FERMENTATION BY STRAIN No. 830 OF MELIBIOSE LIBERATED FROM RAFFINOSE AND THE RAPID FERMENTATION OF PURE MELIBIOSE BY MELIBIOSE-CONDITIONED CELLS

on the surface. It is the only strain which gave confusing results in this technique for determining melibiose fermentation.

The detection of strains which caused a belated fermentation of melibiose from the raffinose solution indicates that for best results a long period of incubation should be used, preferably 3 to 4 weeks, although most strains give definite results within a week to ten days.

To summarize, the technique described has the advantage over methods based on quantitative evolution of carbon dioxide since it requires less apparatus and no chemical titration. It does not require contact of the fermenting medium with mercury, as in the method used by the Dutch school, for contact with mercury over a long period of time might be deleterious to the cells. This would be of especial importance in the case of strains producing a belated fermentation of melibiose. Raffinose is to be preferred to melibiose in this test, since some strains of slow melibiose-fermenting yeasts may not attack pure melibiose if

they produce an alkaline reaction in the medium, whereas they will ferment the sugar as it is liberated from raffinose. Only in the case of one strain which had not utilized all the sugars within 50 days was the technique inadequate.

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ON THE ENRICHMENT AND PURIFICATION OF CHROMOGENIC SPORE-FORMING ANAEROBIC BACTERIA¹

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Brief details of a method for the successful enrichment of chromogenic clostridia have been published (McClung, 1942b). Publication of more complete details of the technique is stimulated by numerous inquiries concerning the method.

Baker (1935) has made the statement that obligate anaerobes are never pigmented. This is not true as evidenced by a list of pigmented species reported in the literature: *Bacterium melaninogenicum* of Oliver and Wherry (1921), *Micrococcus gazogenes* of Hall and Howitt (1925), *Bacillus venturelli* of Carbone and Tomasi (1925), the eight species of Carbone and Venturelli (1925), *Clostridium felsineum* of Carbone and Tombolato (1917), *C. xanthogenum* of Graaf (1930), *C. roseum* of McCoy and McClung (1935), *C. carbonei* of Arnaudi (1937), *Bacillus haumani* of Soriano (1930), *C. cellulosa dissolvens* of Khouvine (1923), the thermophilic cellulose fermenter of Śnieszko (1933) and the blue pigmented organism of McClung (1942a).

Our interest in the problem centered originally on the study of the characters of cultures similar to or identical with *C. felsineum* which were isolated by chance (McClung, 1942c). After realizing that this organism is more common in nature than is commonly believed, an investigation was made of methods which would be satisfactory for its isolation. In the course of this study we have encountered not only *C. felsineum* but also another orange pigmented type which seems to be a new species. Three or four other types in which a yellow pigment is a distinctive character also have appeared. The blue pigmented organism, enriched in one sample, has been noted previously (McClung, 1942a). The characteristics of the various groups of cultures will be the subject of future publications. Perhaps it should be emphasized that all cultures are obligate, spore-forming anaerobes; no growth ensues unless strictly anaerobic conditions are maintained.

METHODS

Samples: We have studied mainly samples of mud from fresh water lake bottoms, and mud from small streams in which decaying organic material was present. A number of strains were obtained from cultivated and virgin soils.

Media: Although various media have been tested, the medium found to be

¹ This problem has been supported, in part, by a grant from the Research Fund of the Graduate School of Indiana University. The author desires also to acknowledge the kindness of the Department of Agricultural Bacteriology of the University of Wisconsin for the provision of research facilities and samples in the summer of 1941 during which time a portion of these studies were in progress.

most useful was five percent corn meal mash. This has unique properties especially suited to this problem: (1) pigmentation of all cultures so far isolated is pronounced on this medium; (2) the medium, being semi-solid, remains anaerobic throughout the extended incubation periods which were shown to be necessary; (3) it requires no seal nor incubation in special anaerobic jars, and thereby frequent examination of each tube is possible; (4) it is inexpensive and easy to prepare. The corn-liver medium of McClung and McCoy (1934) is suitable also, but has the disadvantage that a transfer from each tube which shows growth must be made to plain corn mash to detect pigmentation, as the pigments of certain strains of the yellow group are not readily seen in the darker corn-liver medium.

The plain corn mash is prepared as follows: Place 50 grams of ground white corn meal in a container and add 1000 ml. of tap water. This is heated in freely flowing steam (Arnold sterilizer) for a minimum of one hour during which time the mash is stirred three or four times. After the steaming period the mash is cooled nearly to room temperature which results in an increase in viscosity and this facilitates even distribution during the tubing process. There is some evidence that this cooling aids in the formation of the semisolid layer of starchy material at the top of the medium in the finished product. A stirring device for the tubing funnel is a convenient aid but is unnecessary to success. After tubing the medium in 2 inch columns in sterile plugged tubes (16 mm. x 150 mm.), it is autoclaved for 30 to 40 minutes at 15 lbs. pressure. The longer sterilization time suggested by McClung and McCoy (1934) is no longer believed necessary. This medium should be a white, semisolid mass in which the coarse particles of the corn settle to the bottom leaving a 1 to 2 cm. layer of starchy material at the top which acts as a seal.

For purification, the plating medium most frequently used has been yeast-infusion starch agar. The yeast infusion may be prepared from yeast cells (10 per cent infusion of yeast cells, usually starch-free yeast, autoclaved for 3 hours and the supernatant infusion collected) or from dehydrated yeast extract (0.5%). To the yeast infusion 0.3% soluble starch is added and 15 (shred) or 20 (granular) grams of agar are added for solidification purposes. This is sterilized for 30 minutes at 15 lbs. pressure.

Inoculations: Tubes of the above medium are inoculated with 1.0 ml. of decimal dilutions (from 1-10 to 1-10,000) of the sample. These dilutions are prepared in sterile tap water. At least two tubes should be inoculated from each dilution; one of these is incubated at 37°C. and the other at room temperature (27-30°C.). Following these inoculations the original dilutions are pasteurized (80°C. for 20 minutes), and a second set of tubes are inoculated. If time and materials permit, it is wise to inoculate more than one tube for each temperature as duplicate tubes from a dilution do not always give the same enrichments, and, thus, with the larger number of tubes there is less chance of missing a positive sample.

The tubes incubated at 37°C. are examined daily for seven days at which time the negative tubes are discarded. Whenever a positive tube is encountered it is

removed and incubated at 30°C. for several days to induce sporulation. Purification procedures may then be initiated. The tubes incubated at room temperature are examined twice each week for a minimum of 5 to 6 weeks as it has been noted frequently that, with certain enrichments, pigmentation did not occur until the third or fourth week of incubation.

PURIFICATION OF CULTURES

Unfortunately, at the present time no one method of purification may be recommended as superior to all others. Due to the nature of the sample the enrichments are contaminated by a wide variety of other organisms, and the successful isolation of a pure chromogenic species from the original corn mash tube taxes the imagination and ingenuity of the worker. Several factors are responsible for this. One of the most vexing of these is the fact that pigmentation in the early transfers of a purification routine may be delayed for ten to fourteen days, whereas a pure culture, once obtained, will show good pigmentation within one to three days. Progress is necessarily slow. Another problem, which relates particularly to the *C. felsinium* group, is that rapid subculturing tends to diminish pigmentation. There is some evidence that this is concerned with the necessity of sporulation in the maintenance of the vigor of the culture.

The most obnoxious of the contaminating organisms, as may be expected, are spore-forming aerobic types. These are able to grow sufficiently in the various anaerobic conditions tested to dispell any hope of eliminating them on the basis of oxygen requirements. In plates or agar tubes the formation of microcolonies of the aerobes may go undetected unless careful observation of the plates is made with the aid of a microscope (high power dissecting microscope or a low power of the usual microscope). Until this was discovered, the continual presence of an aerobic contaminant in cultures picked from apparently well-isolated colonies remained a mystery. The use of crystal violet to suppress these contaminants was not of value. In serial transfers in glucose tryptone broth and in corn mash to which the dye had been added, the chromogenic organism invariably was lost before the aerobic contaminant.

The *C. felsineum* type offers another problem. Surface growth takes the form of rapidly spreading, radial, finger-like colonies which may extend several centimeters from the center. This type of colony frequently overruns small colonies of other organisms. Deep colonies do not show the spreading, but, unless the seeding is very dilute, bubbles of gas from these colonies split the agar and this is undesirable in the purification procedure. Light seeding is not the answer to this question as the organism sought may not appear if it is in the minority in the population.

Another difficulty concerning the yellow types is that certain of these may not show distinct pigmentation on the agar media which have been used for plating. For example, one type which shows a fair yellow-green pigmentation in corn mash shows only a light cream colony on starch agar following extended incubation. The picking of colonies then becomes a hit or miss proposition. This is

not true of all yellow types as with some the color is pronounced and may even cause trouble by diffusing throughout the medium, rendering it difficult to determine which colonies are pigmented.

Attempts at single cell or single spore isolations by micromanipulation technique have not been made since the chances of success seemed poor. The chief anaerobic contaminants in these enrichments usually are non-pigmented butyric-acid-producing types. Dilution series of an impure enrichment reveal these to be present in greater numbers per unit volume than the pigmented type. Since the two types are essentially similar in morphology of vegetative cell and clostridium, it is expected that a great number of positive single spore subcultures would be necessary before a pigmented culture would be encountered. The low percentage of positive subcultures reported for other anaerobic species makes this method of isolation appear unsuitable for a quick, successful purification.

Suggested method for purification

The above paragraphs were included to illustrate the difficulties encountered in the purification of the cultures which were found in the enrichments. The suggested method for purification to be described leaves much to be desired but represents the most successful system that we have been able to devise. Corn mash tubes which show any degree of pigmentation following inoculation, as discussed earlier, are incubated at about 30°C. for at least 10 days. Spore stains at this time should reveal, among the many organisms, the large oval spores and clostridial forms typical of the butyric acid and butyl alcohol types. A 1-10 dilution of the culture is heated at 80°C., and 1.0 ml. samples are withdrawn at 2 to 5 minute intervals over a 20 to 30 minute period. These are subcultured in plain corn mash and the tube, which upon incubation at 30°C. shows pigmentation, and which represents the longest heating period, is chosen for further subculturing. When spore stains from this tube show the typical forms a similar heating procedure is instituted. This process should be repeated 3 or 4 times before plating is attempted.

The next step involves the plating technique. Qualitative dilution plates (loop dilutions) are prepared in yeast-infusion starch agar, and these are incubated in an anaerobic environment. Clay top plates incubated in an oat jar (McClung, McCoy and Fred, 1935) have been satisfactory. After an incubation period of at least 7 days at 30°C., the plates are examined for pigmented colonies. If these occur, either one or both of the following procedures form the next step in the purification routine. If the colonies appear well isolated, and this is rare except with the *C. felsineum* group, they are picked to tubes of liver broth with liver tissue or to corn liver medium (McClung and McCoy, 1934). Positive subcultures are transferred to plain corn mash to reveal pigmentation. If this occurs, the subculture is replated to test anaerobic purity, and glucose agar slants are streaked to test aerobic purity. When the pigmented colonies are not well isolated on the original plate, a second plating is done immediately, using as inoculum the pigmented colony suspended in sterile tap water. By this means

pure colonies often may be obtained. If both of these methods fail, the heat-shocking technique should be continued.

DISCUSSION

Using the methods outlined above, successful purification of approximately 75 strains of chromogenic anaerobes has been accomplished. In the latter phases of the problem it was not uncommon to obtain a positive enrichment from each of a group of mud samples. Although the non-pigmented anaerobes predominate in such samples, it would seem that the chromogenic types are far more common than has been believed, and it may well be that they occupy an important place in the anaerobic population of the soil and mud wherein decomposition of complex organic material is proceeding. The ability of *C. felsinium*, and others that we have isolated, to decompose pectins is worthy of mention.

SUMMARY

Details are given of a technique which has been developed for the enrichment and purification of chromogenic species of obligately anaerobic bacteria. This involves the inoculation of heated and unheated dilutions of mud into plain corn mash. Following the appearance of pigmentation serial cultures are made in which the inocula are subjected to pasteurization. Final purification is accomplished by plating in yeast-infusion starch agar.

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THE ACCELERATING EFFECT OF SUBLETHAL HEAT ON SPORE GERMINATION IN MESOPHILIC AEROBIC BACTERIA¹

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In their early studies on disinfection, Koch, Gaffky and Loeffler (1881) reported that anthrax spores which had been heated to 90° and 95°C. required longer to produce visible plate colonies than unheated spores. Since that time the growth-delaying action of sublethal heat has been recorded by many investigators, both for spores and vegetative cells: Bredemann (1909), for *Bacillus amylobacter*; Weiss (1921), Esty and Meyer (1922), Esty and Williams (1924), Dickson (1928) and Sommer (1930), for *Clostridium botulinum*; Schultz (1940), for mesophilic aerobes; Allen (1923), for sporulating and non-sporulating mesophilic aerobes; Eijkman (1908) and Hershey (1939), for *Escherichia coli*. There is considerable evidence to indicate that the germination of thermophilic spores is not retarded by non-killing heat treatment; Bigelow and Esty (1920), Esty and Williams (1924), and Feier (1927). According to Sommer this also is true for *Bacillus subtilis*. Earlier observations by Williams (1929) lend support to this contention.

As a result of these reports the belief has become firmly established that sublethal heat has either a delaying or negligible action upon the germination of spores; the possibility that heat might provide a stimulus to germination has been generally overlooked. Eckelmann (1917), in a discussion of the causes of the heat-inhibition of spores, suggested that in some instances stimulation of germination might occur but offered no experimental proof. Allen noted that heat-shock reduced the subsequent generation time of one sporing culture, but it is our observation that a factor may have no influence upon the time required for spores to become heat-labile, and yet may change the rate of subsequent vegetative proliferation.

In this paper we will show that germination of the spores of the mesophilic aerobes may be consistently accelerated by exposure to heat through a wide range of temperatures. Attention has been given to some of the factors which exert an influence upon this reaction. Because of its potential usefulness in the processing of food and other materials this phenomenon possesses more than academic interest.

METHODS AND MATERIALS

The test organisms were the following: *Bacillus megatherium* (N. R. Smith # 696), *Bacillus cereus* (# 369, # 720 N. R. Smith), *Bacillus subtilis* (A.T.C.C.

¹ Presented at a meeting of the Washington Branch, Society of American Bacteriologists, Washington, D. C., February 23, 1943.

#6051, #6634), *Bacillus cohaerens*, *Bacillus fusiformis* (Bureau Dairy Industry Collection), CC (National Canners Association #4149), #9499 (National Canners Association #9499). CC is closely allied to *Bacillus mesentericus*. The position of #9499 in the genus is not known. The plating medium was nutrient agar of the following composition: Difco peptone, 5 g.; Difco beef extract, 3 g.; sodium chloride, 5 g.; glucose, 3 g.; agar, 13 g.; water, 1,000 ml. pH 7.0. The broth was similar, except for the omission of agar. The evaporated milk was prepared from whole milk concentrated 2 to 1 and was not sterilized before use. Total bacterial counts of the evaporated milk prior to use varied between 300 and 700 per ml., of which less than 5 per ml. survived heat at 85°C. for 10 minutes.

The spores were produced on plain nutrient agar slopes; when sporulation was complete, the growth was washed off with distilled water, filtered through cotton, and centrifuged. The water was decanted and the washing process repeated twice. When clumping occurred the clumps were largely broken up by moderate shaking of the suspension with small glass beads. The concentrated stock suspensions thus prepared, practically 100 per cent spores, were plated to determine purity and count and were then held at 6°C. until used.

Germination was determined by the following procedure: A small quantity of the diluted stock suspension of spores was seeded into the test medium and the two thoroughly mixed. The uniformly dispersed suspension of spores was then divided into a series of equal portions, one of which, the control, received no preincubation heat, and was stored at 8°C., while the several remaining portions were preheated at selected temperatures for various periods. These, together with the unheated control, were then adjusted to and incubated at 37°C. for 3 to 5 hours. When the preheating medium was water or buffer solutions, small equal volumes of the heated spore suspensions were subseeded into glucose broth prior to incubation. Following incubation, all samples were heated at 85°C. for 10 minutes in order to kill the spores which had become heat-labile during the incubation interim. Subculturing of the final heated suspensions was carried out in glucose agar plates which were counted after 48 hours at 37°C. Assumption by the spores of the heat-lability characteristic of vegetative cells was accepted as evidence of germination. There was no appreciable change in the pH of the cultures during incubation.

The suspensions were heated in 8 ml. quantities in pyrex tubes in a stirred glycerol bath equipped with a thermo-regulator, which maintained the desired temperature $\pm 0.5^\circ\text{C}$.

EXPERIMENTAL

Germination of spores as affected by preincubation heat

It was observed early in this study that the effect of heat upon the germination of spores is materially influenced by the medium in which they are heated. Four different heating mediums were used.

In table 1 is shown the effect of heat upon the germination of spores, heated and incubated in glucose extract broth, when the suspensions were preheated

at 85°C. as indicated, then held at 37°C. for three hours, and finally heated at 85°C. for 10 minutes and plated. Under these conditions preincubation heat accelerated the germination of 7 of the 9 cultures; stimulation was greatest with *B. fusiformis*, *B. megatherium*, CC, and 9499. The data recorded in the lower part of the table were obtained when the spores received heat equivalent in amount to that of the upper part, except that all of the heat was applied at the end of the incubation period. The data, therefore, at 4-10 and 0-14, 8-10 and 0-18, etc., are directly comparable, in that each series received an equal amount of heat, differing only in the time at which it was applied. Differences in the counts of comparable series are a direct measure of the heat-induced acceleration or retardation of germination. With 7 cultures, preincubation heat mate-

TABLE 1

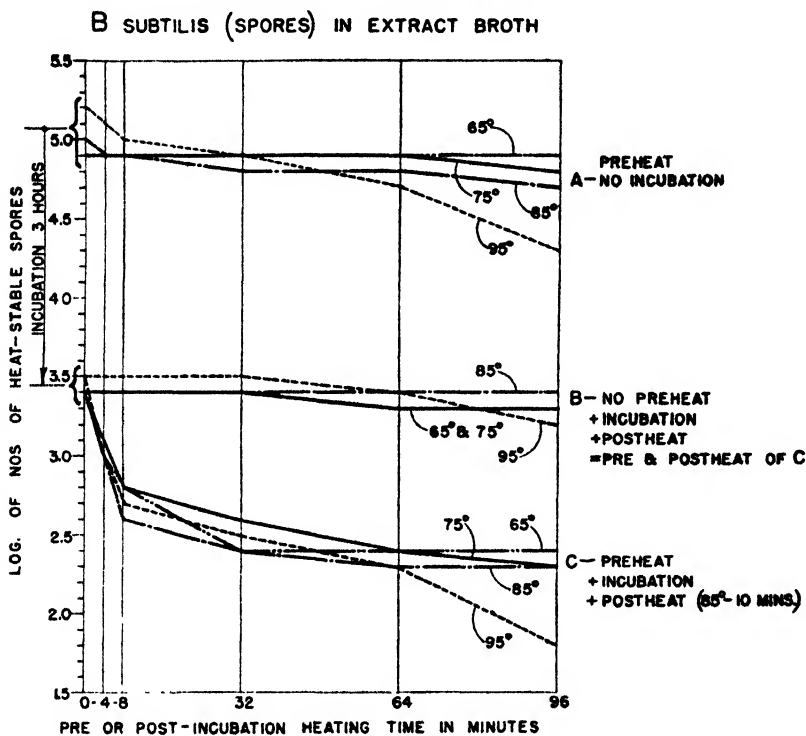
Effect of heat upon the germination of spores when heated and incubated in glucose-extract broth

PRE-INCUBATION HEAT	POST-INCUBATION HEAT	VIABLE SPORES AFTER INCUBATION FOR 3 HOURS WITH PRE- AND POST-INCUBATION HEAT AT 85°C. FOR THE PERIODS INDICATED								
		<i>B. megatherium</i>	<i>B. subtilis</i> (6051)	<i>B. subtilis</i> (6634)	<i>B. cohaerens</i>	<i>B. fusiformis</i>	<i>B. cereus</i> (369)	<i>B. cereus</i> (720)	CC	9499
		(70,000)	(82,000)	(95,000)	(96,000)	(91,000)	(155,000)	(190,000)	(98,000)	(130,000)
minutes	minutes	per ml.	per ml.	per ml.	per ml.	per ml.	per ml.	per ml.	per ml.	per ml.
0	10	7,500	220	2,640	120	32,000	100	120	36,000	120,000
4	10	2,100	110	1,400	220	2,200			27,000	12,000
8	10	290	70	400	220	1,600	800	550	17,000	5,200
32	10	180	80	240	75	400	1,300	450	3,300	2,100
64	10	33	80	190	20	400	250	820	800	2,000
96	10	15	80	180	15	300	8	240	400	2,700
0	14	5,900	130	2,700	110	30,000			37,000	110,000
0	18	4,200	140	2,700	120	29,000	100	140	35,000	130,000
0	42	3,000	140	2,200	130	11,000	10	6	30,000	120,000
0	74	2,000	110	2,400	100	7,000	20	3	32,000	110,000
0	106	900	130	2,300	100	1,400	2	0	31,000	120,000

() Number of viable spores per ml. immediately after inoculation.

rially accelerated the change from the heat-stable to the heat-labile condition. With *B. cohaerens* stimulation was apparent only after the longer heating periods. The anomolous reaction obtained with the two strains of *B. cereus* is of interest in itself, and in connection with the behavior of this species in subsequent heating mediums. The data for 9499 require explanation. Our observations clearly demonstrate that a large proportion of the spores of this organism are normally dormant and will not germinate in the absence of preliminary heat treatment. Eight minutes of heating at 85°C. was found to increase the number of colonies which subsequently developed on plates, by about 35 per cent, as compared with the unheated control; thus, with this culture, when part of the heat is applied before incubation the resulting count reflects the effects both of accelerated

germination and heat activation of normally dormant spores, but with all the heat applied after incubation normal germination plus heat activation of dormant spores is indicated. In the latter instance the slight change in the number of heat-stable spores in relation to their initial concentration indicates an essential balance between normal germination and heat activation. The relatively constant values for CC, *B. cohaerens*, and *B. subtilis* (2 strains), when all of the heat was applied after incubation, suggests a correlation between delayed germination and heat-resistance. Although, as will be shown (figs. 1 and 2), the longer heating periods kill substantially more of the unincubated spores than the shorter heating periods, this is not reflected in any significant change in the number of



spores that do not germinate (table 1, lower portion), indicating that spores of low heat resistance are not among those which exhibit slow germination.

In table 2 are shown the results obtained when the spores were heated in distilled water, and then transferred to glucose extract broth for incubation and postincubation heating. Under these conditions preliminary heat stimulated the germination of 5 of the 8 cultures. *B. cereus* (2 strains), retarded in their germination by heat in glucose broth, were somewhat stimulated by the same degree of heat applied to water suspensions. The reverse was true for *B. cohaerens* and the two strains of *B. subtilis*.

In another experiment the spores were seeded in evaporated milk, preheated

as shown and incubated for 5 hours, followed by the usual mild heating. As may be seen (table 3), preincubation heat under these conditions accelerated the germination of every culture. For eight minutes of preheating the minimum reduction in heat-stable spores over the unheated control was about 40 per cent, while the maximum was approximately 95 per cent.

Why relatively mild heat should speed the germination process is not known, but a possible clue is afforded by the work of Cook (1931) and Tarr (1933). The former found that the boiling of spores of *B. subtilis* prior to their seeding in tryptic medium reduced the lag in oxygen uptake over that of unheated spores; the latter showed that heat at 80°C. for 30 minutes greatly increased the dehydrogenase and oxidase activity of spores of *B. subtilis* suspended in a phosphate

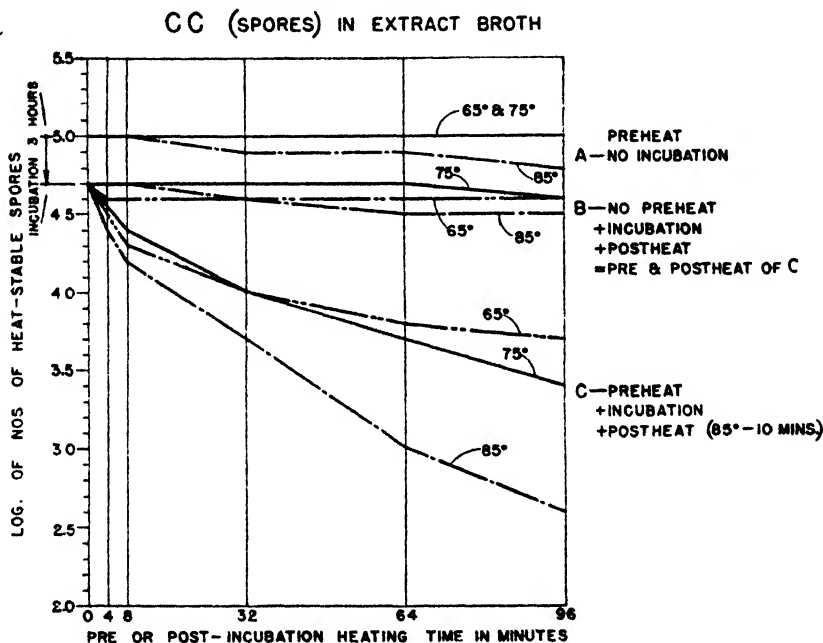


FIG. 2

buffer solution. Eighty degrees C. was more effective than 60°, 70°, or 90°. Tarr further showed that the increased oxygen uptake resulting from preheating the spores was maximal when the pH of the buffer suspension was 6.5, whereas it dropped off rapidly below pH 6.0. Prompted by this observation, we studied the effect of heating spores in phosphate buffers of differing pH followed by subculturing the spores in glucose broth at pH 7.0. The results are shown in table 4. The preincubation concentrations of spores, both with and without preheating are included, since the sporicidal action of heat at 85°C. varied with pH. It is evident from these data that the buffer medium is not a particularly favorable heating medium for demonstration of the heat effect. For *B. subtilis*, greatest acceleration of germination occurred at pH 6.3, with little demonstrable effect at pH 5.2, which accords with the effect of preheating of *B. subtilis* at 80°C. upon

the activity of respiratory catalysts reported by Tarr. With CC, preheating at pH 5.2 seemed to produce the greatest acceleration of germination. For *B. megatherium* the pH of the heating medium had no significant influence upon the preheating effect.

The importance of adequate nutritional stimuli for the subculture of heated spores has been previously emphasized by the publications of Süpfle and Dengler

TABLE 2

Effect of heat upon the germination of spores when heated in distilled water and incubated in glucose-extract broth

PRE-INCUBATION HEAT	VIALE SPORES AFTER PREHEATING AT 85°C. AS INDICATED PLUS INCUBATION FOR 3 HOURS PLUS 85°C. FOR 10 MINUTES								
	<i>B. megatherium</i>	<i>B. subtilis</i> (6051)	<i>B. subtilis</i> (6634)	<i>B. cohaerens</i>	<i>B. fusiformis</i>	<i>B. cereus</i> (369)	<i>B. cereus</i> (720)	CC	9499
	(75,000)	(85,000)	(90,000)	(95,000)	(90,000)	(40,000)	(160,000)	(101,000)	(135,000)
minutes	per ml.	per ml.	per ml.	per ml.	per ml.	per ml.	per ml.	per ml.	per ml.
0	28,900	110	2,700	84	7,900	57	2.7	31,000	123,000
4	7,200	470	6,000	300	4,400	7.0	3.0	21,000	48,000
8	5,100	700	9,000	410	3,600	4.3	0.6	11,000	44,000
32	700	800	10,000	670	1,800	1.3	0.6	10,000	40,000
64	100	1,600	9,000	740	430	0	0.6	7,000	26,000
96	60	1,800	8,000	860	280	0.3	0	8,000	22,000

() = concentration of viable spores per ml. immediately after inoculation.

TABLE 3

Effect of heat upon the germination of spores when heated and incubated in evaporated milk

VIALE SPORES AFTER PREHEAT AT 85°C. FOR THE PERIODS INDICATED, PLUS INCUBATION FOR 5 HOURS, PLUS 85°C. FOR 10 MINUTES

PRE-INCUBATION HEAT	<i>B. megatherium</i>	<i>B. subtilis</i> (6051)	<i>B. subtilis</i> (6634)	<i>B. cohaerens</i>	<i>B. fusiformis</i>	<i>B. cereus</i> (369)	<i>B. cereus</i> (720)	CC	9499
	(70,000)	(82,000)	(95,000)	(96,000)	(91,000)	(155,000)	(190,000)	(98,000)	(130,000)
	per ml.	per ml.	per ml.	per ml.	per ml.	per ml.	per ml.	per ml.	per ml.
0	51,500	370	7,200	3,500	4,000	4,400	3,200	44,000	123,000
8	30,300	200	2,300	1,100	720	770	970	11,000	15,000
32	9,500	200	1,200	600	500	350	380	2,500	6,000
64	4,800	170	900	400	300	300	420	400	5,400

() = concentration of viable spores per ml. immediately after inoculation.

(1916), Morrison and Rettger (1930) and Curran and Evans (1937). The present results show that the heating medium, independently of the subculture medium, influences the subsequent behavior of heated spores.

A clearer picture of the action of sublethal heat upon the germination of spores is obtained when the data for a given culture (*B. subtilis* # 6634) are represented graphically as shown in figure 1. As may be seen, temperatures ranging from 65° to 85°C. with no incubation produced little change in the number of viable

spores. At 95°C. the direct killing was material when the heating was continued longer than 32 minutes. The 5.0 to 3.5 drop on the log scale at B represents the normal germination of the unheated spores during the incubation period of 3 hours. The much smaller number of heat-stable spores in series C shows that preheating materially accelerated the germination of the spores so treated. In general the preheating at 85°C. produced greater stimulation of germination than higher or lower temperatures. The lower counts obtained at 95°C., where the preheating time exceeded one hour, merely reflects the greater direct killing effected by this temperature. Figure 2 shows a similar series of curves for CC wherein the accelerating effect of preheat was more pronounced than for *B. subtilis*, and in which the differences produced by the changes in preheating temperatures were much wider. Data for 95°C. are omitted, since the highly lethal action of this temperature rendered the data of little significance.

TABLE 4

The germination of spores in glucose broth with 3 hours of incubation at 37°C. after previous heating in buffer solutions of differing pH followed by post incubation heating of 85°C. for 10 minutes*

	pH 5.2				pH 6.3				pH 7.4			
	No incubation		3 hours at 37°C.		No incubation		3 hours at 37°C.		No incubation		3 hours at 37°C.	
	No pre-heat	Pre-heat	No pre-heat	Pre-heat	No pre-heat	Pre-heat	No pre-heat	Pre-heat	No pre-heat	Pre-heat	No pre-heat	Pre-heat
<i>B. subtilis</i> #6634 ...	94,000	67,000	1,380	1,050	92,000	86,000	1,180	220	100,000	87,000	840	220
<i>B. megatherium</i>	69,000	48,000	37,000	23,000	77,000	66,000	38,000	21,000	78,000	67,000	34,000	22,000
CC	106,000	84,000	30,000	10,000	97,000	86,000	22,000	12,000	78,000	65,000	21,000	18,000

* 85°C. for 8 minutes.

Persistence of the heat effect

We have shown that appropriate heat treatment accelerates the germination of bacterial spores if favorable cultural conditions are promptly provided. In the belief that light might be shed on the mechanism of the reaction, some observations were made upon the persistence of the preheat effect. This was determined by the delayed cultivation of spores in broth following their heating in distilled water. The data shown in table 5 indicate that heat-altered spores do not quickly revert to their former condition in the absence of favorable growth conditions. With the exception of *B. megatherium*, the heat-induced effects, whether of retardation or acceleration, showed little change throughout the observation period.

The advantage of preheating spores in reducing germination time

It was deemed of interest to ascertain the advantage of preheating spores as measured by the time required for heated and unheated spores to attain comparable germination levels. In an effort to accomplish this preheated (85°C. for 8 minutes) and unheated spores were suspended in glucose broth and incubated

at 37°C. for 3, 8, 17, and 24 hours, following which the heat-labile cells were killed by treatment at 85°C. for 10 minutes. The data are shown in table 6. As may be seen, the germination of unheated spores proceeded very slowly after

TABLE 5
Effect of delayed cultivation of heated spores upon their germination*

CULTURE	PRECULTIVATION TREATMENT	NO INCUBATION	TIME AFTER PREHEATING OF CULTIVATION† FOLLOWED BY HEATING OF 85°C. FOR 10 MINUTES		
			Immediately	1 day	7 days
		<i>per ml.</i>	<i>per ml.</i>	<i>per ml.</i>	<i>per ml.</i>
<i>B. cohaerens</i>	85°C.—10 min. No preheat	90,000	570	340	290
		104,000	140	92	90
<i>B. subtilis</i> #6634	85°C.—10 min. No preheat	71,000	5,500	4,400	4,600
		79,000	1,100	1,170	920
<i>B. megatherium</i>	85°C.—10 min. No preheat	88,000	8,100	14,000	15,000
		95,000	28,000	27,400	29,700
CC	85°C.—10 min. No preheat	106,000	26,000	22,000	21,000
		91,000	51,000	54,000	51,000
9499	85°C.—10 min. No preheat	150,000	26,000	22,000	25,000
		113,000	102,000	107,000	116,000

* Heating medium, distilled water.

† 3 hours in glucose broth.

TABLE 6
The germination of preheated spores during 3 hours of incubation in glucose broth compared with that of unheated spores over a 24-hour incubation period

CULTURE	INITIAL CONCENTRA- TION OF SPORES	VIABLE SPORES AFTER INCUBATION FOR TIMES INDICATED, PLUS POST INCUBATION HEAT 85°C. FOR 10 MINUTES					
		Heated before in- cubation	Not heated before incubation				
			3 hours	3 hours	8 hours	17 hours	24 hours
			per ml.	per ml.	per ml.	per ml.	per ml.
<i>B. cohaerens</i>	92,000	210	110	28	26	31	
<i>B. subtilis</i> # 6634.....	95,000	350	2,600	1,340	460	443	
<i>B. megatherium</i>	78,000	260	8,000	4,400	3,000	2,500	
CC.....	95,000	18,000	36,000	21,000	16,000	15,000	
9499.....	127,000	5,800		106,000	102,000	98,000	

the first three hours; little or no change in the number of heat-stable cells occurred during the last 8 hours. In general, three hours of incubation preceded by mild heating was superior to 24 hours of incubation without preheat as measured by the reduction in number of heat-stable spores. A longer preheating

of the *B. cohaerens* suspension would probably have brought the data on this culture into essential agreement with those of the other cultures (table 1). Although the descending order in the number of heat-stable spores speaks against resporulation, a separate experiment was performed to test this point. It was found that there was no resporulation during the 24-hour observation period with *B. subtilis*, *B. megatherium* and 9499. With *B. cohaerens* and CC resporulation occurred, but in numbers so small as to have no significant influence upon the results.

DISCUSSION

It is well known that many chemical poisons, including nearly all salts, will stimulate bacterial development if suitably diluted in a nutrient medium (Rahn, 1932; Topley and Wilson, 1937). Likewise there is considerable evidence that sublethal doses of ultraviolet radiation may stimulate bacterial growth (Coblentz and Fulton, 1924; Hollaender and Duggar, 1938). That there should be an analogous operation of this principle for temperatures beyond the natural range of growth of bacteria seems reasonable. For certain fungus spores this has been clearly demonstrated. Thus the ascospores of *Ascobolus* and *Neurospora* are normally dormant and will germinate only after they have been heated (Dodge, 1912; Shear and Dodge, 1927). Goddard (1935) later found that the heat necessary for the activation of these spores is correlated with a large increase in respiration (O_2 consumed). Evidence of the stimulative action of sublethal heat upon the respiratory enzymes of bacterial spores has been cited (Cook, 1931, and Tarr, 1937). It was shown by the latter that the real effect of heat was to reduce the time required for the attainment of a constant velocity of oxidation. It is, perhaps, significant that both the maximum stimulation of enzymic respiratory activity (Tarr) and the acceleration of germination occur at essentially the same temperature levels. Strain 9499 was noteworthy in that greatest stimulation followed preheating at 95°C. The greater heat-resistance of this species suggests that there may be a correlation between thermal resistance and the optimum preheating treatment essential for maximum acceleration of germination.

The observation that sublethal heat may speed rather than delay the germination of spores is contrary to general belief and the recorded observations of the investigators previously cited (page 513). The reasons for this discordance are not apparent; however, mention should be made that previous investigators based their conclusions on the relative periods required for heated and unheated spores to form visible colonies on plates—a process which involves both germination and vegetative multiplication.

The results obtained with the different heating mediums are of interest in connection with an earlier observation by Morrison and Rettger (1930a). These authors found that the spores of a spoilage organism when heated in water and subcultured in broth exhibited the familiar "skips" with a consequent ill-defined thermal death-time. When, however, the same suspension was heated in evaporated milk and subcultured on standard agar plates "skips" were absent and

the thermal death-time sharply defined and apparently constant. The similarity of this reaction with that observed for *B. subtilis* and *B. cohaerens* (tables 2 and 3) is significant and points to a better understanding of the irregularities frequently encountered in heating tests.

Although only a small proportion of spores is affected by preheating, these potentially dormant forms are nevertheless of primary economic significance, since delayed germination and high resistance are intimately associated phenomena; the factors which make for maximum resistance seem also to render the spores less responsive to nutritional stimuli.

SUMMARY

Sublethal preincubation heating of aerobic spores may be used to accelerate their germination.

Temperatures in the 65–95°C. range are effective accelerators of spore germination. In general, greatest acceleration was obtained with a preheating treatment of 85°C. for 8 to 10 minutes.

The effect of preheating upon spore germination is influenced by the nature of the medium in which the spores are heated and incubated. With some species preheating may accelerate or retard germination, depending on the nature of the preheated medium.

Preincubation heat accelerated spore germination in 7 out of 9 cultures heated and incubated in glucose broth.

Preincubation heat accelerated spore germination in 5 out of 8 cultures heated in distilled water, and incubated in glucose broth.

Preincubation heat accelerated spore germination in 9 cultures (no exceptions) heated and incubated in evaporated milk.

The effect of preincubation heat upon spore germination is influenced by the reaction of the preheated medium. The optimum pH for acceleration of germination apparently differs with the species.

When spores were heated, and held in distilled water, their altered capacity for germination was still manifest after 1 week.

In general three hours of incubation preceded by mild heating was equivalent or more than equivalent to 24 hours of incubation without preheating as measured by the reduction in numbers of heat-stable spores.

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MACHINE FOR SHELL FREEZING SMALL VOLUMES OF BIOLOGICAL PREPARATIONS

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The preferred method for preserving certain types of biological preparations, such as yellow fever vaccine, consists in quick-freezing followed by desiccation under high vacuum from the frozen state with subsequent storage *in vacuo* or an atmosphere of dry nitrogen (Sawyer *et al.*, 1929; Bauer *et al.*, 1940; Hargett *et al.*, 1943). In the Rocky Mountain Laboratory of the United States Public Health Service, yellow fever vaccine is put up in pyrex ampoules of the type shown in figure 1. The stem of the ampoule measures 7 by 60 mm. and the body 25 by 90 mm. Although ampoule capacity approximates 28 ml., vaccine content is limited to 5 ml. This volume relationship was established to permit the rapid freezing of the vaccine in the form of a thin-walled hollow cylinder preparatory to desiccation. This method of freezing has been denoted "shell freezing." Ampoules of similar design and reduced capacity are employed to receive lesser volumes.

The requirement of a rapid and efficient means of shell freezing the vaccine following distribution into ampoules led to the development of the freezing machine to be described. Efficiency, availability, and cost factors dictated the employment of carbon dioxide ice as the refrigerant and 95 per cent ethyl alcohol as the circulation medium.

THE MACHINE

Because of the difficulty of obtaining metal and parts, brought about by war requirements, construction was of locally available materials. An alcohol-resistant paint replaced the to-be-desired chromium plating. Construction was of metal with cork insulation.

One photograph (fig. 1) and two drawings (figs. 2 and 3) show general construction and most details. The unit consists of dry ice chamber A, freezing compartment B, ampoule rotating unit C, ampoule leveling device D, and pump E. The dry ice compartment is closed by snug-fitting cover A₁. Vent A₂ leads from near the top of ice chamber for exhausting carbon dioxide while alcohol is emptied by drain line A₃. The escape of dry ice particles by way of fluid openings in compartment A is prevented by fixed screens. The carbon dioxide ice is contained in wire basket A₄.

The depth of the freezing compartment B is 3½ inches compared to 9½ inches for dry ice chamber A. Refrigerated alcohol is received into B from line E₄ and directed to remote portions of chamber by baffles B₁ and B₂. Alcohol is returned to compartment A from chamber B through the duct formed by the walls of the partition common to A and B. Slot-shaped duct inlet B₃ is shown in figures 1

and 3. The lower lip of the duct inlet is adjustable to permit some alteration of channel mouth. The duct outlet, not shown in figures, is a slot-shaped opening at bottom of chamber A. Retrograde passage through the duct of carbon

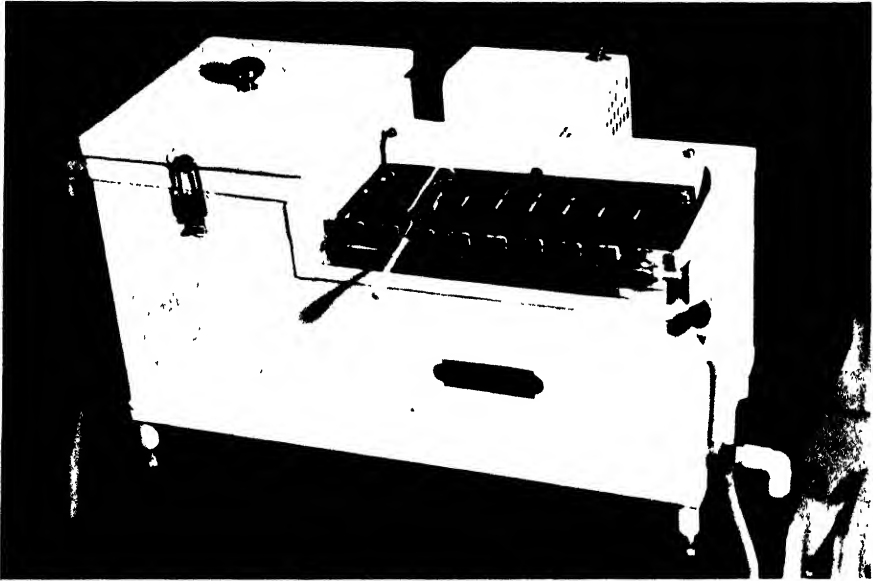


FIG. 1. AMPOULE FREEZING MACHINE WITH ONE 28 ML. AMPOULE IN FREEZING POSITION

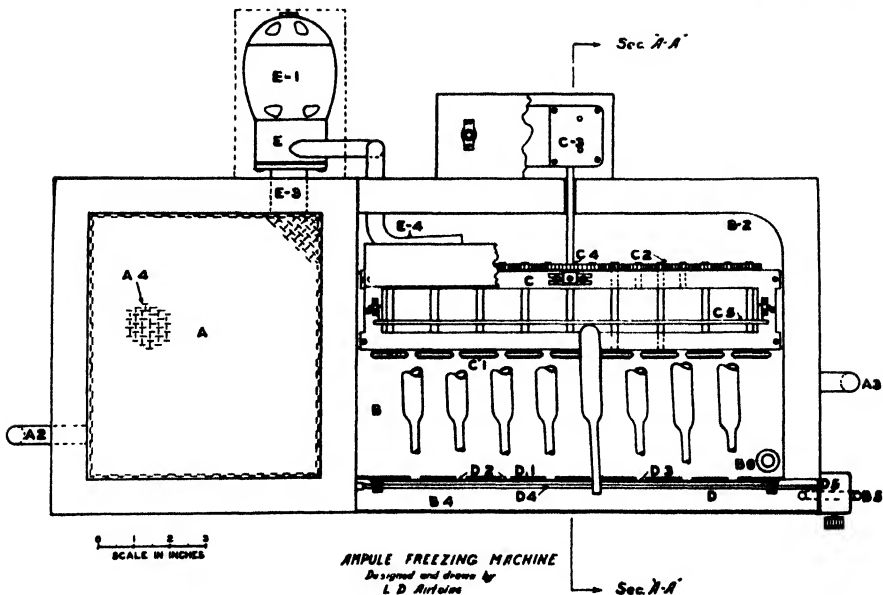


FIG. 2. TOP VIEW TO SHOW GENERAL CONSTRUCTION

Note that long axes of ampoules are slightly non-parallel to drive wheel shafts.

dioxide evolved in A is prevented by the trap construction of the duct. The small quantity of fluid lost through leveling device notches D₂ is collected in trough B₄ and carried away through drain B₅. In emptying the machine of alcohol, compartment B is drained by line B₆.

Ampoule rotating unit C consists of 9 rubber-tired drive wheels C₁ directly connected by shafts to gear train C₂ which is driven by constant speed fractional horsepower motor C₃ via shaft and drive gear C₄. Idler gears are employed so that all drive wheels rotate in the counterclockwise direction. The motor is

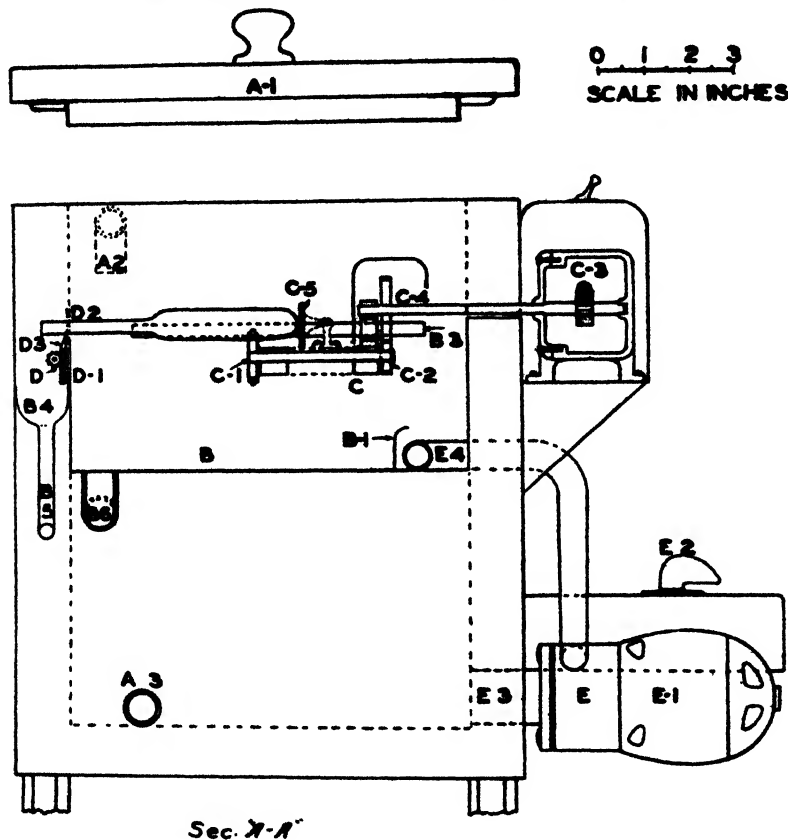


FIG. 3. VERTICAL CROSS SECTION THROUGH FREEZING CHAMBER AND DRIVE MOTOR

fixed to the backside of compartment B, while the drive mechanism is mounted on a chassis secured by brackets within freezing chamber B. Screws at both ends of the chassis permit alignment and leveling. Plate C₅ serves to retain the butt ends of the ampoules which are forced against it by the fact that the axes of the rotating ampoules are slightly non-parallel with the axes of the drive wheel shafts. This is obtained by notches D₂ being placed slightly to the right of a point in line with the axis of the opposite idler gear shafts. The plate is mounted on pivots allowing it to be turned front or back to accommodate

ampoules of varying body length. Figure 1 shows it in the "back" position. Protective guards, which are readily removed, shield drive motor and gears.

Ampoule leveling device D is mounted on front of compartment B. It supports ampoule stems and serves to level ampoules of varying body diameter by elevating or lowering the stems. Fixed plate D_1 contains eight notches D_2 , whose relation to the drive mechanism has been described. Notch margins have beveled edges to reduce drag on rotating ampoules. Movable plate D_3 , whose upper edge is beveled, fits snugly on the front side of fixed plate D_1 and is secured to it by flat-headed pins at either end, passing through slots which permit vertical movement. Adjustment of D_3 is effected by shaft D_4 fitted with fixed pinion gears meshing with gear teeth in the movable plate. The shaft is activated by small worm drive D_5 operated by a thumb screw.

Centrifugal pump E, mounted on backside of compartment A, is powered by a variable speed motor E_1 controlled by a rheostat switch E_2 . Pump intake line E_3 takes off from near the bottom of chamber A, while pump discharge line E_4 terminates within compartment B. This latter line is insulated by a thickwalled snug-fitting rubber jacket.

The machine is fixed in position on a laboratory bench by a threaded steel rod secured to the middle of the underside of the unit and passing into the bench. Adjustment screws on each of the four legs permit ready leveling. A rubber tube attached to vent A_2 conducts carbon dioxide to the floor. Additional tubes connecting to alcohol drain lines A_3 and B_5 pass to a collection bucket under the bench. An electric fan is mounted on the wall in a horizontal position directly above the machine to direct a current of air onto the unit.

To place machine in operation, basket A_4 within compartment A is almost filled with chunks of carbon dioxide ice. Pump E is then set in motion and pre-chilled (minus $60^{\circ}\text{C}.$ to minus $75^{\circ}\text{C}.$) alcohol added so that the fluid level in A is about two inches below the inferior margin of notches D_2 . Cover A_1 is then placed in position to prevent overflow of generated carbon dioxide gas from A. The fan is then turned on to force the carbon dioxide evolved from the alcohol in chamber B to the floor, from whence it is removed by the room exhaust. Removal of carbon dioxide from the vicinity of the freezing chamber is desired to prevent its being drawn into ampoules during freezing.

Alcohol, refrigerated by intimate contact with dry ice in A, is drawn through line E_3 to the pump and impelled *via* line E_4 to freezing compartment B. Return to dry ice chamber is by gravity *via* channel B_3 . Temperature in the freezing chamber is to an extent regulated by flow from A, which is governed by the operational speed of the pump. A temperature of minus $60^{\circ}\text{C}.$ to minus $70^{\circ}\text{C}.$ is readily maintained in the freezing compartment. Height of alcohol in B is so fixed that the inferior 4 to 9 mm. of ampoules in freezing position are immersed. This is accomplished by adjustment of the lower lip of channel mouth B_3 and ampoule rotating unit C.

As many as eight ampoules of the type described, loaded with vaccine and plugged with cotton, are placed in the machine as illustrated with bodies supported by drive wheels and stems by properly elevated leveling device D. The

drive mechanism rotates the ampoules in a clock-wise direction at 116 R.P.M. This speed of rotation insures a constant film of cold alcohol on the non-immersed portions of the ampoules. The 5 ml. of contained vaccine solidly freezes in the form of a thin-walled hollow cylinder, suitable for desiccation, in about 15 seconds. As freezing is completed the ampoules are removed to a holding bath of dry ice and alcohol. Similar style ampoules of varying sizes and content may be "shelled" with equal ease. At termination of freezing, cover A_1 is removed and dry ice taken out by means of container basket A_4 . Alcohol is drained *via* line A_3 .

COMMENT

Employing the 28 ml. ampoules (fig. 1) containing 5 ml. of vaccine each, one operator with the described machine can freeze 1,000 units per hour. The resultant frozen shell is far more perfect and suitable for desiccation than was vaccine frozen by previously employed hand methods. The use of a friction rather than an engagement drive for rotation of ampoules simplifies and accelerates operation.

SUMMARY

One operation in the desiccation method of preserving certain biological preparations, such as yellow fever vaccine, requires that it be frozen rapidly in the form of a thin-walled hollow cylinder. The electrically operated machine described, employing carbon dioxide ice as the refrigerant and ethyl alcohol as the circulation medium, accomplishes this effectively and rapidly, with only a single operator required.

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EFFECT OF LONG ULTRAVIOLET AND SHORT VISIBLE RADIATION (3500 TO 4900Å) ON *ESCHERICHIA COLI*

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INTRODUCTION

The lethal effect of long ultraviolet ($> 3500\text{\AA}$) and short visible ($< 4900\text{\AA}$) radiation on bacteria has been a controversial question for many years. Toxic effects of parts of this region of the spectrum have been reported by Ward (1894), Bayne-Jones and Van der Lingen (1923), and Coblenz and Fulton (1924). Negative results have been reported by Gates (1929), Ehrismann and Noethling (1932), and Bachem and Dushkin (1935). Additional literature is reviewed in a paper by Duggar (1936).

Carefully controlled experiments have made it possible to establish the conditions for the lethal action of the long ultraviolet and near-visible radiation. A study of certain sublethal effects gave an opportunity to distinguish the mechanism of the radiation action of wave lengths shorter than 3000\AA and longer than 3500\AA .

EXPERIMENTAL TECHNIQUE

The bacteria (*Escherichia coli*) were grown on nutrient agar (Difco) slants (20 hours at 37°C .), washed off with physiological salt solution,¹ shaken, filtered through absorbent cotton and centrifuged; and the sedimented organisms were resuspended in salt solution. They were then irradiated in phosphate buffer (pH 6.8), in a concentration of 200,000 to 1,000,000 organisms per ml. Exposures were made in Pyrex culture tubes, which were rotated by a simple mechanism. During the time of irradiation, both the exposed and the control tubes were kept in a constant-temperature bath.

Light sources used were either a General Electric H-6 lamp in a glass jacket or a water-cooled medium-pressure quartz capillary mercury vapor lamp of the Daniels-Heidt type (using 1 to 4 amp. at 150 to 240 volts). The radiation was concentrated, by means of a 500 ml. round-bottom flask filled with a water solution of copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ —2 grams per liter) and a condensing lens, into a constant-temperature water tank with a glass window. The radiation also had to pass through two Corning Glass filters (# 738, 4 mm. and 585, 2 mm.) before entering the tank. (For details see figure 1.)

Spectrograms² of the incident radiation reaching the irradiated tube were taken with a quartz spectrograph, over a wide range of exposures, at the surface of the exposed culture tube. The photographs, even after very extended expo-

¹ Composition of physiologic salt solution: NaCl —3g, KCl —0.2 gr, CaCl_2 —0.2 gr, 100 ml. distilled water.

² The spectrograms were taken by Dr. P. A. Cole of this Laboratory.

tures, showed no trace of radiation shorter than 3500\AA and only very little above 4358\AA in wavelength. Most of the energy was concentrated in the 3650\AA set of lines; next in energy concentration came the 4358 group, and finally the 4046 set and some minor lines. The energy was determined by means of a thermopile connected with a high-sensitivity galvanometer, standardized against a National Bureau of Standards lamp. The exposure technique is very similar to that described by Hollaender, Brackett and Cole (1938).

Samples were removed from control and exposed tubes at certain intervals during exposure; these were diluted with physiological salt solution and plated out with nutrient agar, or incubated in nutrient broth or physiological salt solution and then plated out after certain time-intervals of incubation. At least three plates were poured for each dilution, and incubated for 48 hours at 37°C . All colonies were counted. Each of the counts given in the graphs and tables represent an average of at least three plates.

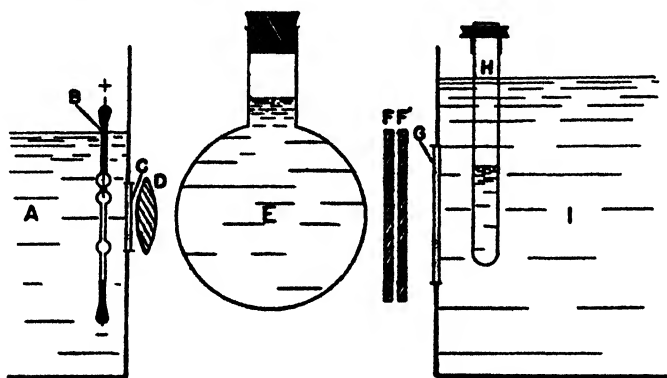


FIG. 1. EXPERIMENTAL ARRANGEMENT FOR IRRADIATION OF BACTERIA WITH λ 3500\AA TO 4900\AA AT CONSTANT TEMPERATURE

A. Water bath for radiation source. B. Capillary mercury vapor lamp. C. Quartz window. D. Condensing lens. E. Round bottom flask filled with dilute solution of copper sulphate. F. Corning Glass filter #738, F' Corning Glass filter #585. G. Glass window. H. Exposed culture tube in rotating device. I. Constant temperature water bath.

RESULTS

A typical killing curve is given in figure 2. This figure also includes a graph showing the killing effect at wave length 2650\AA . Attention is called to two distinct differences between these curves: (1) The incident energy necessary to produce 50 per cent killing at $3500\text{--}4900\text{\AA}$ is 5×10^8 ergs/cm², while the energy necessary to bring about the same proportion of killing at 2650\AA is about 10^8 ergs/cm². It requires, therefore, 10,000 to 100,000 times as much incident energy to kill at $3500\text{--}4900\text{\AA}$ as at 2650\AA . (2) The log survival ratio energy curve approaches a straight line for 2650\AA and is decidedly of the threshold type for the long ultraviolet.

Temperature effect. The bactericidal action of wave lengths shorter than 3000\AA has a temperature coefficient of about 1.1 (Gates, 1930). Radiation of $3500\text{--}4900\text{\AA}$ has a temperature factor of about 1.7 to 2.2 (i.e. 10° rise in tem-

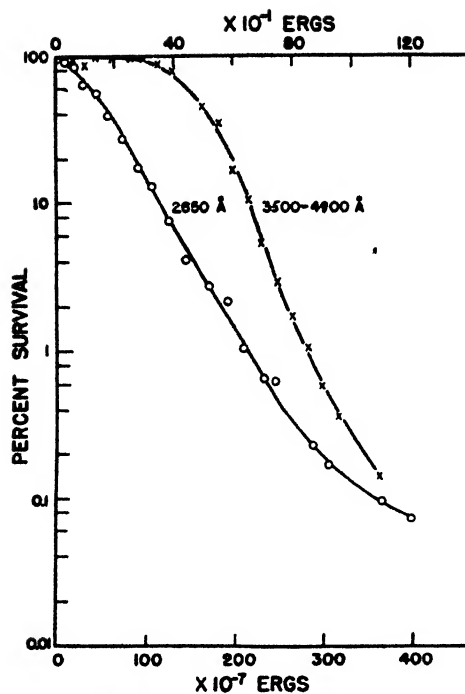


FIG. 2. SURVIVAL RATIO AGAINST ERGS/ORGANISM FOR *E. COLI* IN LIQUID SUSPENSION
Curve 3500 to 4900Å uses upper abscissa and 2650Å curve lower abscissa

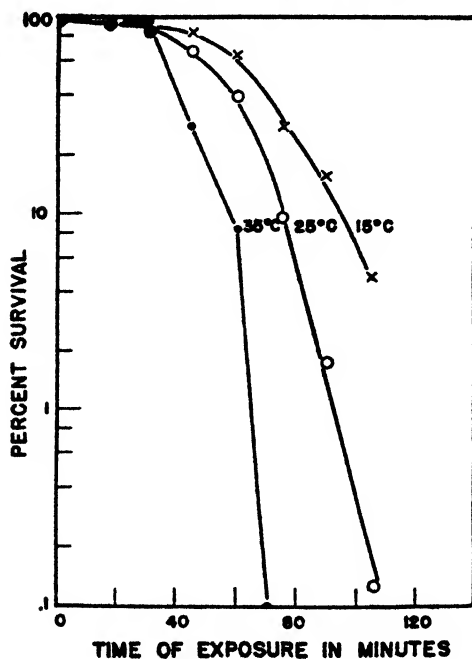


FIG. 3. CHANGE IN SURVIVAL RATIO WITH TIME FOR CONSTANT ENERGY (3500 TO 4900Å)
AT THREE TEMPERATURES

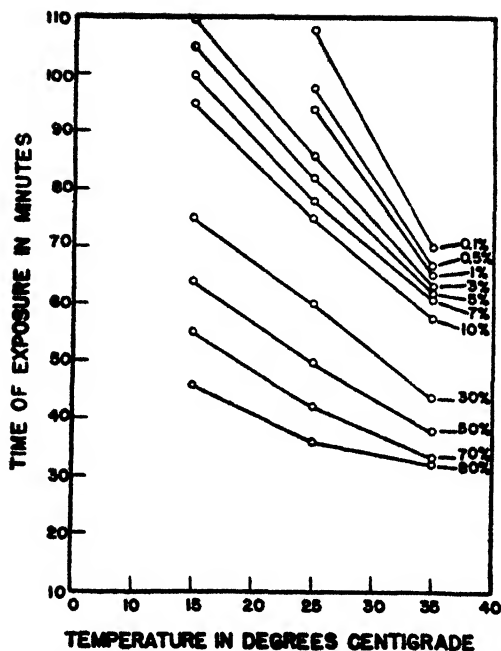


FIG. 4. TIME OF EXPOSURE AT CONSTANT ENERGY (λ 3500 TO 4900Å) PLOTTED AGAINST TEMPERATURE FOR A NUMBER OF SURVIVAL RATIOS, SHOWING THE INCREASING EFFECT OF TEMPERATURE ON LOW SURVIVAL RATIOS

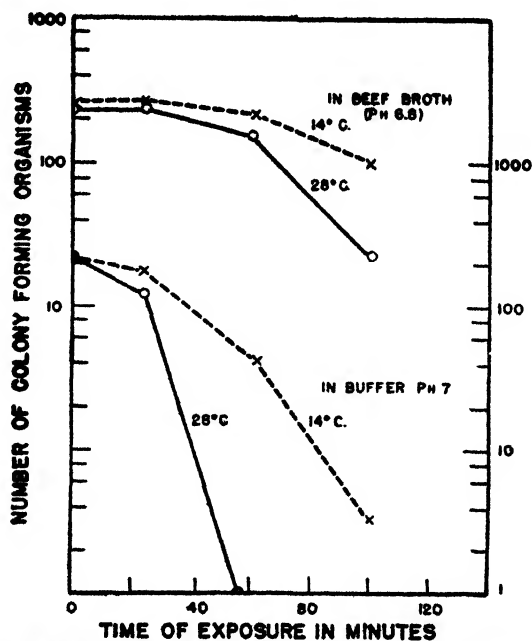


FIG. 5. CHANGE IN THE RELATIVE NUMBER OF COLONY-FORMING ORGANISMS WITH TIME OF EXPOSURE TO 3500 TO 4900Å IN EXPOSED (SOLID LINE) AND CONTROL (BROKEN LINE) CULTURE IN BEEF BROTH (LEFT SCALE) AND BUFFER SOLUTION (RIGHT SCALE)

perature reduces the energy necessary to kill by about one half). No effect of temperature is noticeable at the threshold part of the curve. However, as soon

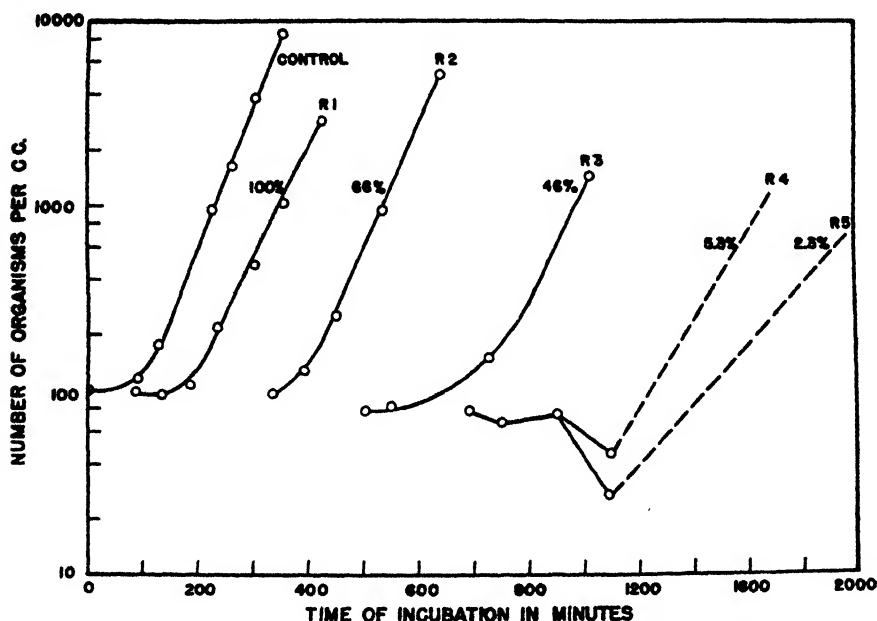


FIG. 6. CHANGE IN THE RELATIVE NUMBER OF COLONY-FORMING ORGANISMS PER CC. WITH TIME OF INCUBATION AFTER IRRADIATION, (WITH λ 3500 TO 4900Å) FOR FIVE SURVIVAL RATIOS AND CONTROL CULTURES

At low survival ratios the growth ratio was less reliable (broken lines). Details are given in the text.

TABLE 1
Effect of 3500 to 4900Å radiation on retarded-growth phase

CONTROL			RUN 2—SURVIVAL RATIO 86 PER CENT		
Time of incubation	No./ml.	Per cent	Time of incubation	No./ml.	Per cent
<i>minutes</i>			<i>minutes</i>		
0	379	100	0	325	100
82	444	117	90	322	102
127	749	176	150	339	104
217	3,560	940	210	289	92
262	5,900	1,556	270	306	94
307	14,500	3,820	330	309	95
367	31,800	8,400	390	420	129
			450	850	260
			525	3,020	930
			630	16,300	5,000

as the lethal action appears, i.e. after 40 minutes of exposure (see fig. 3) there is a very definite temperature effect which increases with the time of exposure. This is shown strikingly in figures 3 and 4.

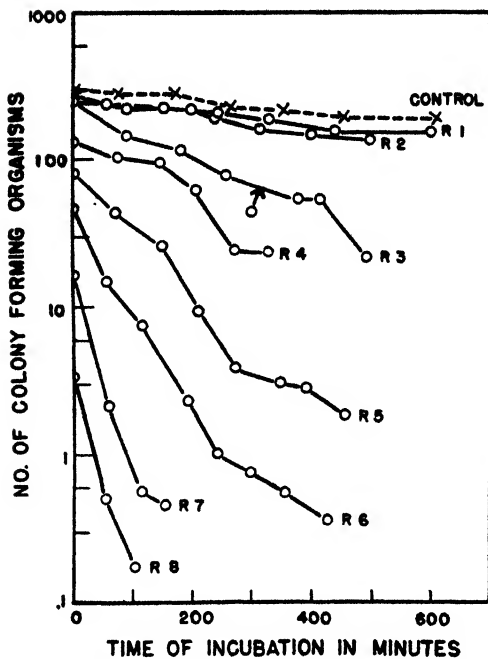


FIG. 7A. RELATIVE NUMBER OF COLONY-FORMING ORGANISMS AGAINST THE TIME OF INCUBATION IN PHYSIOLOGICAL SALT SOLUTION FOLLOWING IRRADIATION (3500 TO 4900Å)

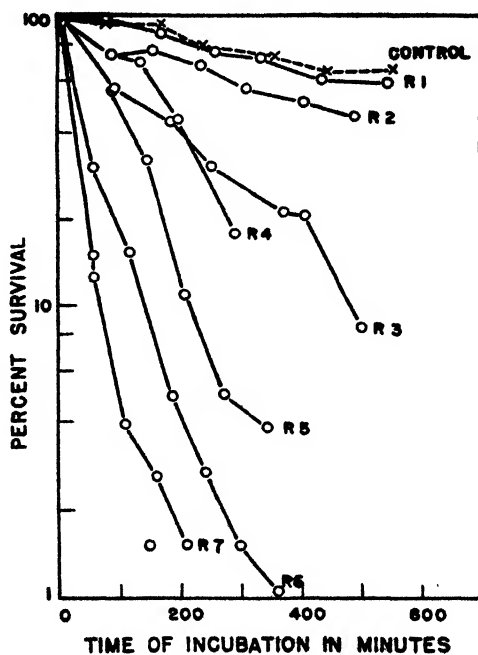


FIG. 7B. SAME DATA AS SHOWN IN FIGURE 7A (BUT) RECALCULATED ON A PERCENTAGE BASIS

The effect of irradiation in beef broth as compared with irradiation in buffer solution is shown in figure 5. Beef broth has a fairly high absorption coefficient in the near-ultraviolet region and thus protects the bacteria suspended in it to a considerable extent. The effect of salt solution is also discussed below.

Extension of "retarded growth phase." A study of the effect of ultraviolet radiation by wave lengths shorter than 3000\AA on the extent of the "retarded growth phase" has provided interesting information concerning the function of the shorter ultraviolet (Hollaender and Duggar, 1938). Essentially the same technique for determining the growth rate was used with bacteria surviving irradiation with 3500 to 4900\AA . The method consists in determining the number of colony-forming organisms by making plate counts immediately after irradiation and at certain intervals of incubation of the irradiated and control organisms in dilute beef broth. A typical set of growth curves is shown in figure 6. Data for the control and for Run 2 of this experiment are given in table 1. Each curve is marked with the survival ratio. The extension of the retarded growth phase begins before any organisms are killed by irradiation. With increasing energy, the delay in the initiation of cell division increases until a certain limit has been reached; after about 99 per cent of the organisms have been killed, no further extension of this phase appears to be possible.

Effect of salt solution. The "extended retarded growth phase" found after incubation in beef broth points to a "weakening" of bacteria that survive 3500 to 4900\AA radiation. As a more definite check on this point, control and irradiated bacteria were suspended in physiological salt solution, and the number of colony-forming organisms was determined immediately after irradiation and at certain time-intervals of incubation. The results of a typical experiment are given in figures 7a and 7b. The control survived incubation in physiological salt solution very well for ten hours. The dying rate of bacteria after irradiation was substantially greater than of the control.

DISCUSSION

Outstanding findings on the lethal action of the region 3500 – 4900\AA as compared with the region below 3000\AA are given in table 2. Ultraviolet photographs of bacteria taken at wave lengths shorter than 3000\AA show considerable detail, whereas photographs taken at the longer wave lengths show only general absorption (Wyckoff and Ter Louw, 1931), indicating that the compounds responsible for the effect of the longer wave lengths are probably diffused throughout the entire cell and are present in such small concentrations that they have no visible effect on the photographs taken. The extended threshold nature of the killing curve suggests the production of some toxic substance, or the destruction of some essential compound in the cell, the effect of which, up to a certain limit, does not permanently destroy the ability of the cell to divide and develop further. The presence of a high-temperature factor often indicates the existence of some secondary process. In general, photochemical reactions have low temperature coefficients. The region below 3000\AA has a low coefficient, indicating that here the lethal effect results from a direct photochemical process. A dif-

fusion process, which might distribute a toxic compound formed by the radiation, would tend to have a high coefficient (see Belehradsek, 1935).

The extensive prolongation of the so-called "lag phase" of the bacteria points definitely to the injurious effect of 3500–4900Å. It appears that this injurious effect is produced in a systematic manner, since a plot of the extension of the "retarded-growth phase," against energy gives a straight line. At the highest energy tested this straight-line relationship breaks down, indicating that the injury eventually becomes too great to permit recovery of the organisms. This injury to the bacteria expresses itself also in a lowered resistance of the organisms when suspended in physiological salt solution. It appears that the bacteria can obtain from nutrient beef broth (Difco) the material necessary for repairing the

TABLE 2
Effects of the 3500 to 4900Å and the 2180 to 2950Å region

	3500 to 4900Å	2180 to 2950Å
1. Shape of killing curve (log survival ratio/energy)	Threshold type	Approaching straight line
2. Energy (incident) for 50% survival ratio	Approximately 2×10^8 ergs/cm ²	5×10^8 to 10^9 ergs/cm ²
3. Temperature coefficient	1.7–2.2	1.1
4. Sublethal effects appear	Before any organisms are killed (in threshold part of killing curve)	After 60 to 90% of organisms are killed
5. Extension of retarded-growth phase for 10% survival ratio	Up to 1000%	50%
6. Toxicity of certain salt solutions can be recognized	At once after irradiation	In 600 minutes at 32°C.
7. Mutation production	No mutations	Mutations produced in fungi and <i>Drosophila</i>

cell, but that it is impossible for repair to take place without a supply of such material from outside the cell, as demonstrated when a suspension of physiological salt solution is used. Very little evidence can be found of the existence of a toxic substance producing the secondary effect observed after irradiation. The relative ease of recovery of irradiated organisms makes such an interpretation less probable, although the possibility cannot be eliminated. More support is available for the explanation that the radiation has destroyed some compound essential for the survival and multiplication of the bacterial cell. The extended retarded growth phase would then be the time necessary for the cell to replenish the essential material destroyed by the radiation. The dying rate in physiological salt solution would imply that the cell was not able to replace from other

cell constituents the compound destroyed by the radiation. In the case of relatively low energy values only a fraction of the "material" was effected by the radiation, and there was sufficient material left to keep the cell alive for a certain time in the non-nutrient salt solution.

A change in the morphology of the cell could be produced directly by the radiation; it also could be a secondary effect following the destruction of essential compounds necessary for the support of the cell. It is possible that in the destruction of an essential compound, toxic substances (see above discussion for limitations) are produced which interfere with the function of the cell, resulting finally in a change of the permeability of the cell wall or a change in some other physical structure of the cell. A relatively slight extension of the retarded-growth phase is also found after irradiation with wave lengths shorter than 3000Å. A possible explanation would be that the major effect of these wave lengths ($< 3000\text{\AA}$) is through absorption in the nuclear material and that a residual effect is produced in the general protoplasm (Knaysi and Mudd, 1943). This latter effect might be similar to the one described for 3500 to 4900Å. (A detailed discussion of these points will be found in a forthcoming publication by Hollaender and Duggar.)

The bactericidal action of ultraviolet radiation of wave lengths shorter than 3000Å is most efficient at 2650Å, close to the wave lengths toward which nucleic acids are most highly absorbent. However, as far as has been tested, nucleic acid has no measurable absorption at wave lengths 3500 to 4900Å. Only relatively few biologically important compounds show definite absorption bands in this region of the spectrum. For instance, riboflavin has a set of bands in this region (Booker, 1939). It is also known that riboflavin becomes toxic after irradiation and is present in bacterial cells. We have added riboflavin to the suspension after irradiation of the bacteria, in the hope of shortening the radiation-produced extension of the "retarded-growth phase," but with no success. In all these experiments special care was taken to keep the bacteria dark after irradiation. Further tests on compounds, particularly respiratory enzymes which might be affected by the radiation, are now in progress.

Effects of irradiation with 3500–4900Å, similar to the ones described here for *E. coli*, have also been observed with other bacteria (*Eberthella typhosa*) also with yeast (Hollaender, Cole, and Brackett, 1938), fungi (Emmons and Hollaender), and nematode eggs (Jones and Hollaender, 1942). It is important to mention that no genetic effect of the range 3500–4900Å has been observed with fungi and *Drosophila* (Hollaender and Emmons, 1941); no systematic study on this point has been undertaken with bacteria.

The region of the spectrum from 3500 to 4900Å is of high intensity in sunlight. For instance, in Washington, D. C. on a clear day in July around noon-time, the intensity of this spectral region is approximately 4×10^4 ergs/cm²/sec. (Smithsonian Physical Tables, 1934). The intensity obtained in our laboratory with artificial sources is 3.5×10^6 ergs/cm²/min. According to these data, *E. coli* would be killed in a standard medium on a clear day at 25°C. in about 1 to 2 hours, if wave lengths below 3500Å were excluded. In tropical sunlight

the germicidal effect of natural radiation would be very high, especially if we consider that radiation below 3500Å and incidental infra-red radiation is added to the 3500–4900Å radiation. We will return in a further publication to the implications of the findings reported in this paper in relation to the hygienic and ecological influence of sunlight.

Since the completion of this manuscript, a report has been published on the effect of natural daylight and sunlight and artificial illumination on streptococci (Buchbinder, Salowey, and Phelps, 1941). Insofar as these studies can be compared with ours, they check quite well.

SUMMARY

1. A method for the irradiation of bacteria with measured quantities of 3500 to 4900Å is described.
2. The effect of the 3500 to 4900Å region on *Escherichia coli* is compared with the effect of radiation between 2000 and 3000Å with respect to the energy necessary to kill, shape of killing curve, temperature coefficient, extent of retarded-growth phase and survival after irradiation in physiological salt solution.
3. Possible mechanisms for the effect of radiation of 3500 to 4900Å are discussed.

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PENICILLIN

ITS USE IN MEDIA FOR THE ISOLATION OF *H. INFLUENZAE* FROM LARYNGEAL CULTURES IN OBSTRUCTIVE LARYNGITIS

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The study of antibiotic substances has assumed a place of great importance in the field of bacteriology in recent years. Fleming (1929) observed the antibacterial action of filtrates of *Penicillium notatum* on gram-positive cocci and suggested its use for the isolation of *Hemophilus influenzae* from mixed cultures. In 1932 Fleming published a further study on the effect of this filtrate penicillin, and of potassium tellurite on various microorganisms, obtained from many different sources. Since that time penicillin has been studied *in vitro* for its antibiotic action on various microorganisms in this country and in England (Bornstein, 1940; Fleming, 1942; Craddock, 1942; Hobby, 1942).

At the Willard Parker Hospital, laryngo-tracheo material from patients suffering from obstructive laryngitis is studied for its bacterial flora. This material is usually abundant in gram-positive cocci, and isolation of *H. influenzae* due to overgrowths of these cocci proved difficult on ordinary laboratory media. Penicillin as used by Fleming was the choice as the basis for selective media for *H. influenzae* studied from this type of material.

The syndrome of laryngo-tracheo-bronchitis was first attributed to *H. influenzae* in 1936 by Lemierre (1936). Recently Alexander *et al.* (1942) discussed the frequency of isolating *H. influenzae* from cases of severe obstructive laryngitis. They stressed the importance of the identification of the causative microorganism in order that proper treatment be instituted. Sinclair (1941) has recommended that routine blood cultures be taken for the isolation of *H. influenzae* as this microorganism will often escape identification in routine throat cultures alone. At the Willard Parker Hospital, *H. influenzae* has been recovered from several patients from the blood and tissues cultured at autopsy from fatal fulminating cases of obstructive laryngitis. However, no consistent bacterial flora was found in routine nose, throat and laryngeal cultures on ordinary laboratory media, taken on our croup service. This has been confirmed by the other workers mentioned above.

In May 1941 a systematic study of the bacterial flora of cultures taken at laryngoscopic examination was undertaken by us. (The clinical analysis of this work is delayed by Dr. Fiegoli's service in the armed forces and will be presented at a later date.) However, it was felt that the bacteriological study should be presented now, as the methods described may have practical applications in other laboratories.

PREPARATION OF MEDIA

A. Preparation of the Penicillin Medium (Fleming)

1. A fairly large inoculum of both spores and mat of a known culture of *Penicillium notatum*, obtained from the American type culture collection, was grown in shallow layers of trypsin digest broth. 75 ml. of this broth in 250 ml. Erlenmeyer flasks gave the best yields of penicillin.
2. The culture was kept at room temperature (20°C.) for eight to ten days depending on the rate of growth. A heavy mat with many spores and high alkalinity of the medium was used as evidence of penicillin production.
3. The reaction was adjusted to approximately pH 6.8 with 3% HCl using phenol red as an indicator.
4. The cultures were then filtered through an 'N' Berkfeld filter and the filtrate (penicillin) was tested for potency. These tests were necessary because the inhibitory values of the active principle in such crude filtrates is not always the same. Therefore the amounts of penicillin necessary to inhibit gram-positive cocci without interfering with the growth of the influenza bacillus vary with each preparation. The test was performed as follows:

Serial dilutions of the penicillin were made in 10 ml. of beef heart infusion broth as follows: 1:50, 1:100, 1:500, 1:1000. 0.1 ml. of an eighteen-hour broth culture of *Staphylococcus aureus*, previously adjusted to contain 1×10^7 microorganisms per ml., McFarland scale, was added to each tube. Failure to develop turbidity after twenty-four hours at 37°C. was accepted as evidence of inhibition. Inhibitory action was determined by the least amount of penicillin necessary to inhibit the growth of one million microorganisms. The penicillin when stored in the refrigerator maintained its potency for six to eight weeks.

5. This inhibitory dilution of penicillin was then tested for its effect on a known *H. influenzae* culture. The proper dilution of penicillin, horse blood and beef heart agar were mixed in the ration of 10:5:100. A stock *H. influenzae* culture was streaked on plates poured from this preparation and incubated at 37°C. for twenty-four hours. If good growth was obtained this dilution was used in the study. These plates were designated as "penicillin plates." Whole blood was used in preference to "chocolate" blood because the plates were clearer and easier to read.

B. Preparation of Blood Agar Plates:

Beef-heart infusion broth with 1.5% agar was melted and 5% sterile citrated horse blood was added and plates poured. These were designated as "blood agar plates."

C. Sodium Oleate Plates:

5 ml. of a 2% sterile sodium oleate solution (neutral) and 1 ml. of red blood cells (horse) was added to each 100 mls. of beef extract agar and plates poured. These were designated as "sodium oleate plates."

This medium was recommended by Avery in 1921 for the isolation of *H. influenzae*. By the action of the sodium oleate the growth of many gram-positive cocci present in nose and throat secretions is inhibited. As the medium had been used previously in our hospital, it was included in this study for purposes of comparison.

NOTE: These culture plates were stored in the refrigerator of the examining room and were therefore always available.

PROCEDURE FOR OBTAINING CULTURES

Of the patients admitted to our croup service during the period May 1941 to January 1943, 119 with obstructive laryngitis were included in this series. On admission direct laryngoscopic examination was done on all the patients to determine the type of laryngeal involvement (to be reported). During this examination material was obtained from the larynx by swab and it was immediately

cultured on the three media described. The cultures were then incubated at 37°C. for eighteen to forty-eight hours depending on growth and then examined. Identification of *H. influenzae* was made on the basis of colony formation, stained smears and requirements for growth of the 'x' and 'V' factors, by transplants to plain beef-heart agar. Serological classification was done only in the last few cases as the rabbit-typing serum for *H. influenzae* had not been available before.

In this series of 119 cases, *Hemophilus influenzae* was recovered from 68 (57.1%) patients. In all, 357 cultures were examined, as each specimen was cultured on the three media as described. The recovery of the microorganism from each medium is tabulated in table 1. In thirteen instances *H. influenzae* was recovered from the penicillin plates only, and in nine instances only from the sodium oleate plates. 48.5% of the blood agar plates were positive in contrast to the 86.7% positive findings from the penicillin medium and 80.8% on the sodium oleate plates. The use of the selective media and our diligent search may account for such a high percentage of positive cultures. The causal relation between the finding of this microorganism and the clinical course of obstructive laryngitis will be presented in another paper.

TABLE 1
Effect of selective media on recovery of H. influenzae

	PENICILLIN PLATES	SODIUM OLEATE PLATES	BLOOD AGAR PLATES	TOTAL	PER CENT
Positive for <i>H. influenzae</i>	59	55	33	147	41.3
Negative for <i>H. influenzae</i>	60	64	86	210	59.7
Totals.....	119	119	119	357	100

Despite the apparent parallelism in the successful recovery of *H. influenzae* from the penicillin and sodium oleate plates, we found a great discrepancy in the appearance of their growth. Very few of either the gram-negative or positive cocci grew on the penicillin plates so that the small dew-drop-like colonies of *H. influenzae* were readily discernible and easily recognized. This proved to be very advantageous when serological tests were done. The "quellung" reaction was done directly from the original culture by emulsifying a few of the colonies in saline and then adding the serum. This original culture can also be used in the same manner when testing for the patient's antibodies as described by Alexander (1942).

The sodium oleate plates had many more of the other microorganisms and although they were simpler to read than the blood agar plates, more fishings were necessary to obtain *H. influenzae* in pure culture. The blood agar plates had to be examined very carefully and frequently only in a small portion of the plate were any of the small dew-drop-like colonies of *H. influenzae* found. Table 2 gives the analysis of the growths on the three types of media.

We were able to isolate many microorganisms other than *H. influenzae* from

these laryngeal cultures. *Neisseria catarrhalis* was the most frequently found on all media. These gram-negative cocci were found on 236 of the 357 plates examined. As neither the sodium oleate nor the penicillin media completely inhibits this organism, this was not unexpected. However, these cocci were found in pure culture on 13 of the blood agar plates, 16 of the sodium oleate plates and 41 of the penicillin plates.

Streptococcus viridans was the next in frequency to be recovered; 151 plates being positive and of these nine were in pure culture. *Staphylococcus aureus* was encountered in only 68 of the cultures and this also was recovered in pure culture

TABLE 2

Analysis of growth on 3 media of the 68 cases positive for H. influenzae

	PENICILLIN PLATES		SODIUM OLEATE PLATES		BLOOD AGAR PLATES	
	Number	Per Cent	Number	Per Cent	Number	Per Cent
<i>H. influenzae</i> —pure cultures.....	22	37.2	5	9.1	0	0
<i>H. influenzae</i> —one other microorganism ...	34	57.7	32	58.2	8	24.2
<i>H. influenzae</i> —two other microorganisms...	3	5.1	18	32.7	25	75.8
Totals.....	59	100.0	55	100.0	33	100.0

TABLE 3

Microorganisms other than H. influenzae isolated from cultures (all media)

MICROORGANISM IN	MIXED CULTURES		IN PURE CULTURE	
	Number	Per Cent	Number	Per Cent
<i>N. catarrhalis</i>	236	66.1	70	19.6
<i>Streptococcus viridans</i>	151	42.3	9	2.5
<i>Staphylococcus aureus</i>	68	19.0	9	2.5
Hemolytic streptococcus.....	13	3.6	1	0.3
Pneumococcus.....	8	2.2	1	0.3
<i>C. hoffmani</i>	4	0.2	0	0

in nine instances. Hemolytic streptococci, pneumococci and diphtheroids were rarely found. Table 3.

The relation of the bacteria cultured from the nasopharynx to the severity of the clinical picture of obstructive laryngitis will be discussed in a subsequent paper. However it may be mentioned at this time that in our experience the most severe cases show the growth of *Hemophilus influenzae*.

SUMMARY

1. The addition of crude penicillin to blood agar plates provides a simple but excellent medium for the isolation of *Hemophilus influenzae* from mixed cultures usually obtained from the larynx.

2. Typical growths of *H. influenzae* were obtained from the penicillin media in

practically pure cultures and were readily recognized. It was found that these cultures can be used without further transplants for serological identification and for testing patient's antibody.

3. The ease of preparation of these penicillin plates, using stock laboratory media for a base, makes its use convenient in a busy diagnostic laboratory.

We wish to express our appreciation to Drs. Vera B. Dolgopoul and Jerome L. Kohn for their help in preparing this paper and to Dr. William Kershaw and members of our resident staff who obtained the laryngeal specimens used in this study. Mrs. Jennie Berrell gave invaluable technical assistance in carrying out the details of the laboratory work.

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BACTERIOLOGIC PROCEDURES IN SANITARY AIR ANALYSIS

WITH SPECIAL REFERENCE TO AIR DISINFECTION

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Evidence, amassed from diverse sources (Wells, Wells and Mudd, 1939) proves the importance of air-borne infection. The classic work of Laidlaw and his associates (Dunkin and Laidlaw, 1926; Andrewes and Glover, 1941) upon air-borne spread over considerable distance, of the viruses of dog distemper and influenza, Lurie's (1930) experiments on tuberculosis, dust-borne between animals in separate cages, observations on air-borne surgical infection by Hunt (1933) and Meleney, (1935), Cruickshank's (1935) studies of air-borne infection of burns, the Colebrooks' (1935, 1936) analysis of the rôle of nasopharyngeal organisms in puerperal infection, and the work of Allison (Brown and Allison; Allison and Brown 1937) on streptococcal infection in fever wards and of McKhann (1938) on nosocomial infection in children's wards, are examples of the variety of evidence accumulated in recent years. Conversely, the effect of radiant disinfection of air in reducing surgical infection (Hart, 1936; Overholt and Betts, 1940) and cross-infection in pediatric wards (del Mundo and McKhann, 1941; Robertson, Doyle and Tisdale, 1943), a nursery (Rosenstern, 1942) and an orphanage (Barenberg, *et al.* 1942), and in the environmental control of epidemic spread of contagion in schools (Wells, Wells, and Wilder, 1942), now provides experimental evidence of the importance of air-borne infection. Improved bacteriologic procedures in sanitary air analysis have also reinterpreted Flugge's theory of droplet infection (Wells, 1934; Wells and Stone, 1934), proved quantitative inhalation of droplet nuclei infection to the lung (Wells and Lurie, 1941), demonstrated habitual exchange during winter months of respiratory flora among aggregations occupying enclosed atmospheres (Wells and Wells, 1936), and measured the sanitary inadequacy of present ventilation practice and the potentiality of air disinfection in control of dynamic spread of air-borne infection (Wells and Wells, 1943). Elements of three types of procedure presented to the Committee on Ventilation and Atmospheric Pollution of the Industrial Hygiene Section of the American Public Health Association by the Subcommittee on Bacteriologic Procedure (1937-1943) can now be integrated into a consistent system. (References to these reports will be indicated by year only under the heading "APHA" and original references given in the reports will be repeated only when cited for a different purpose.)

1. *Sanitary survey of inhabited atmospheres.* Rapid methods of collecting

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particles from large volumes of air enable the routine sampling necessary to obtain statistically significant indices of sanitary ventilation.

2. *Experimental studies of bacteria suspended in controlled atmospheres.* The mechanics of air-borne infection and control by ventilation have been studied bacteriologically in experimentally controlled atmospheres.

3. *Measurement of sanitary ventilation.* By quantitative sampling of test organisms added to atmospheres under different ventilating conditions, the hygienic importance of air disinfection has been demonstrated.

Settling rate. The density of bacteria laden particles (D = number per cubic foot) multiplied by settling velocity (V_g = feet per minute) determines settling rate (A = count per sq. foot per minute). If, for example, $V_g = 1$, then D particles will settle upon a Petri dish (1/15th sq. ft.) in 15 minutes and the settling rate of larger particles ($V_g > 1$) or smaller particles ($V_g < 1$) will be proportionately greater or less. The atmospheric density of particles of given size depends upon the rate at which they are lifted into or removed from the air. Since greater effort is required to lift larger particles which settle faster, and since smaller particles which remain longer in air are produced with greater difficulty,³ actual size distribution under uniform conditions tends toward a dynamic equilibrium where mean settling velocity becomes relatively constant (table 1). Within normal limits of variation, then, the Petri dish count, the simplest and for some purposes the most significant determination, is proportional to bacterial concentration ($D = AV_g$) (APHA 1942).

Volume settling. Particles settle rapidly from small confined volumes (Winslow, 1908), but in a closed room the density is progressively reduced by settling, while the rate of deposit remains constantly proportional to the residual density, a relation leading to the rate of decrease in both density and rate of deposit,

$$\log_e D/D_0 = -V_g t/H$$

The logarithm of the residual density, expressed as a proportion of the original density, is a linear function of time (t) when settling velocity (V_g) and room height (H) are constant; or of settling velocity when room height and settling time are constant.

If A_t represents the number of particles which settle on unit area in a given time, then

$$A_t H = D_0 - D = D_0 - D_0 e^{-V_g t/H}$$

or $A_t H \approx D_0$

as settling velocity and time increase and chamber height decreases. Thus $D_0 e^{-V_g t/H}$ becomes a small correction term where time and settling velocity are large and chamber height is small (A.P.H.A., 1942).

³ Thus the work done in grinding powders increases out of all proportion to their fineness, and the velocity of air required to atomize liquids similarly increases without limit as droplets become very small. Just as deposits of water-borne sand tend toward uniformity, so does the equilibrium between carrying power of air and rate of deposition tend to classify air-borne particles.

TABLE 1
Mean settling velocity (V_g) of bacteria laden dust

SOURCE	NUMBER OF SAMPLES	V_g (FEET PER MINUTE) ^a
Outside air ^b		
Near laboratory.....	14	1.67
Near Textile Mills.....	14	25
Textile mill air ^b		
Dusty (carding, etc.).....	17	2.43
Settled (spinning, etc.).....	17	0.91
Humidified (weaving, etc.).....	14	0.42
Hospital air		
Clinic (children, Boston) ^c	23	1.66
Cubicle Wards (infants, Philadelphia) ^c	27	1.14
Operating rooms		
Boston ^c	8	2.04
Pittsburgh ^d		
Air-conditioned.....	76	1.56
Not air-conditioned.....	76	1.32
Iowa City ^e		
General surgery.....	108-64 ^(f)	1.59
Head surgery.....	80-46 ^(f)	0.83
Orthopedic surgery.....	69-36 ^(f)	1.47
Delivery rooms, Iowa City ^e	41-28 ^(f)	1.41
Halls		
Philadelphia ^c	3	1.33
Iowa City ^e	38-16 ⁽ⁱ⁾	2.22
Orphanage air (Philadelphia) ^c		
Nursery.....	6	1.71
Play-room (1-2 year children).....	6	5.26
Dormitory		
Army barracks used as ward ^f		
Morning.....	3	2.93
Evening.....	3	2.00
Sneeze infected air ^g	11-8 ⁽ⁱ⁾	1.06
Droplet nuclei from atomizer ^h	150	0.03

^a Area count (per sq. ft. per min.)/volume count (per cu. ft.).

^b Wells, W. F., and Riley, E. C. An investigation of the bacterial contamination of the air of textile mills with special reference to the influence of artificial humidification. *J. Indust. Hyg. and Toxicol.*, **19**, 513, 1937.

^c Wells, W. F. Sanitary Air Analysis. Unpublished paper read before A.P.H.A., Detroit, Oct. 11, 1940.

^d Cook, W. L. Report on Air-conditioning in Surgery. Univ. Pittsburgh, 1940.

^e Macdonald, K. Quantitative bacterial analysis of the air of operating rooms of a general hospital. *Am. J. Hyg.*, **31**, 74, 1940.

^f These figures were obtained from investigations carried out by the Commission on Cross Infections in Hospitals of the Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, and are cited by permission of the Surgeon General.

^g Bourdillon, R. B., Lidwell, O. M., and Lovelock, J. W. Sneezing, and disinfection by hypochlorites. *Brit. Med. J.*, **1**, 42, January 10, 1942.

^h Phelps, E. B., and Buchbinder, L. Studies on microorganisms in room environments. I. A study of the performance of the Wells air centrifuge and of the settling rates of bacteria through the air. *J. Bact.*, **42**, 321, 1941.

Number of volume and area samples, respectively.

Air centrifuge. The air centrifuge is essentially a sedimentation chamber within which settling velocity is increased by centrifugal force, fixed height

(radius) and settling time (flow) determining an operating constant for the machine. This constant, experimentally determined for smallest bacteria-bearing nuclei gives

$$D = B/(1 - e^{-12r_s}),$$

as the formula of normal machine performance (Phelps and Buchbinder, 1941; Wells, 1942), where (B) is the centrifuge count per cubic foot.

For particles settling faster than one-half a foot per minute, the correction term (e^{-12r_s}) becomes insignificant, and the formula of performance reduces to $D = B$. The centrifuge count thus approximates true density for most particles encountered in surveys summarized in table 1, and if the correction term cancels in ratios used to determine bacterial changes under experimental conditions, it would seldom be required in practice (A.P.H.A., 1942).

Operation. The air centrifuge combines three functions in one operation: pumps a measured quantity of air through a collecting chamber; collects particles in measured quantities of media; or plants them directly on solid nutrient media ready for incubation and enumeration. Solid media must be stiffened by addition of 7-10 grams of agar per liter to the ordinary formula, and in removal from the machine and in incubation, the tubes should be kept horizontal. Dehumidification of the incubator air (by calcium chloride) retards growth of troublesome spreaders and counting may be facilitated by special apparatus (A.P.H.A., 1939). Dilution methods also may be adapted with measured quantities of liquid, the centrifuge serving as a pipette for collecting bacteria from measured quantities of air (A.P.H.A., 1941).

Impingement. The principle of the Owens dust counter has recently been applied to bacterial air analysis (Bourdillon, Lidwell and Thomas, 1941). Particles from a high velocity jet impinge upon a moving agar surface, 2 mm. from the nozzle. The narrow slit nozzle radial to a rotating Petri plate delivers one cubic foot a minute under negative pressure of eleven inches of water. The efficiency of the slit sampler, as it is called, depends upon scrupulous adherence to the dimensions given by the authors, and, if so designed and operated, will remove particles of bacterial dimensions. Fulfillment of these exacting requirements is facilitated by ingenious contrivances neatly built into the apparatus. Where adequate suction is available, this provides a simple, compact and accurate collecting device. Auxiliary equipment required for operation, however, adds to the weight of equipment carried into the field.

Arbitrary combinations of impingement with gravity settlement and with electrical precipitation have also been proposed (Hollaender and DallaValle, 1939; Berry, 1941). Neither settling time nor velocity of approach are adequate to remove small particles, and interpretation depends upon empirical calibration of each device.

State of suspension. Since state of atmospheric suspension reflects the conditions responsible for the presence of particulate matter in air, settling velocity may distinguish sources of bacterial pollution. The sanitary behavior of coarse dust particles also differs significantly from that of aerosols. Mechanical removal of particulate matter by purification devices or by the filtering mechanisms of

the nasal passage is more effective against larger particles, but chemical or radiant disinfection may be more effective against smaller particles which penetrate more readily to the lung. Settling velocity, therefore, as measured by the ratio of

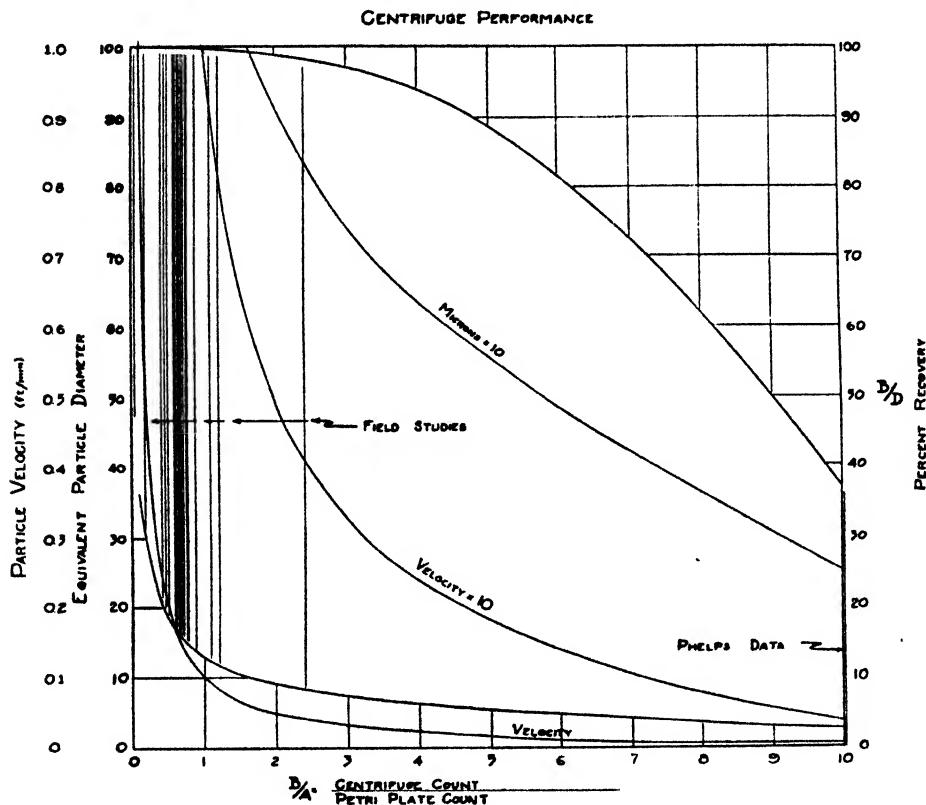


CHART I. AIR CENTRIFUGE PERFORMANCE

$$\text{Per cent recovery} = 100 B/D = 1 - e^{-12V_g}$$

where the constant 12 is derived from Phelps data (Phelps and Buchbinder, 1941) assuming turbulent flow (Wells, 1942).

$$\text{Particle velocity} = V_g = A/D$$

Field study data taken from Table I; equivalent particle diameter calculated from Stokes law (curve "velocity $\times 10$ " magnifying ordinates tenfold).

$$\text{Equivalent diameter (microns)} = 13.3 \sqrt{V_g}$$

(Curve "Microns $\times 10$ " magnifying ordinates tenfold).

A = Area count (per sq. ft. per min.)

B = Centrifuge count (per cu. ft.)

D = Density (particles per cu. ft.)

V_g = Settling velocity under gravity (ft. per min.)

volume to area count, serves in the interpretation of bacterial analysis of pollution and purification of air. The ratios of centrifuge tube to plate count, under various ventilating conditions are shown on chart 1, together with indicated particle size and percentage recovery.

It is apparent that dust particles rather than droplet nuclei usually dominate bacterial air counts. In normal respiration these generally harmless saprophytic organisms from decomposing organic matter are filtered out in the nasal passages. Though not proportionately represented in the count, and numerically insignificant, nuclei from evaporated droplets derived from tissues which may be infected are of major hygienic importance. Their detection provides a more serious problem of bacteriologic procedure than the measurement of air cleansing from dust.

Bacteria laden particles. Rarely under field conditions will the number of bacteria-laden particles represent the total number of bacteria present in air. The chance that dust fragments bear single organisms is remote, and particles of pulverized dirt are more likely to carry hundreds of bacteria. If bacterial clumps of decomposing matter collected in liquids are shaken apart before planting, the number of colonies will greatly exceed the number obtained by direct precipitation on agar. The ratio of counts from solid and liquid media collections thus become a bacterial index of air dirtiness.

Dust may, on the other hand, have greater sanitary significance in wards where the sick are gathered together, and where cross infection of the nose and throat by hemolytic streptococci is not uncommon. Thus, the liquid method of collection may provide a sensitive index of air cleanliness in hospitals. Bacteria collected by the Petri filter method, adopted by the American Public Health Association (Committee on Standards for the Examination of Air, 1917), are washed into sterile water preliminary to planting and several direct methods of air washing have also been proposed (Rettger, 1910; Palmer, 1916; McConnell and Thomas, 1925; Robertson, Bigg, Miller and Baker, 1941; Wheeler, Foley and Jones, 1941; Moulton, Puck and Lemon, 1943).

Sanitary air analysis. The total count, therefore, offers little direct evidence of air-borne infection, nor can bacteriologic procedures hope to recover the few pathogenic organisms in the huge volumes of indoor air breathed per winter, which account for the universal spread of epidemic respiratory disease. Sanitary interest in such procedures rests upon ability to evaluate the defensive barriers against the environmental spread of air-borne infection. Just as the sanitary hazard from drinking water is judged by the number of coliform bacteria of intestinal origin, so does the number of respiratory streptococci in the air offer a basis for estimating hazard of respiratory contagion. Parallel procedures of analysis can be applied to air bacteria collected in liquids, if media adaptable for identification of alpha streptococci are substituted.

Bacteria from 10 cubic feet of air are equally distributed into ten tubes of lactose proteose No. 3 broth containing an indicator (brom-thymol blue) and incubated at 37° for twenty-four hours. Laboratory routine may be simplified by use of circular batteries of Wasserman tubes dispensing with manipulation of cotton plugs (Wells, 1942). Streptococci of the *S. salivarius* type form acid in this medium, but so also do staphylococci which are abundant in inhabited atmospheres. Acid-forming organisms are therefore transferred to gentian-violet blood agar (0.00005 per cent gentian violet in protease No. 3 blood agar)

which inhibits staphylococci. Tubes showing acid after twenty-four hours, and those which do not yield streptococci on the plates, are streaked after forty-eight hours. Recovery of streptococci from a majority of cubic foot volumes tentatively indicates inadequate ventilation (APHA 1942). The dilution method requires less skill than isolation of alpha hemolytic streptococci collected directly on blood agar (2 per cent blood in protease No. 3 agar), but with experience the rusty halo can be recognized even among the large numbers of other organisms obtained in heavily contaminated atmospheres.

Measurement of sanitary ventilation. The principal sanitary contribution of bacteriologic procedures has so far been experimental. Study of the behavior of air-borne pathogenic microorganisms in controlled atmospheres has unveiled a mechanism of spread of contagion and disclosed effective means of control. The effect of physical and chemical agents upon the viability of air-suspended microorganisms and the effect of state of suspension upon the invasion of the respiratory tract can be determined by quantitative laboratory techniques for infecting and disinfecting air. By amplification of tests under ventilating dimensions with a standardized index organism the practical performance of sanitary ventilation can then be interpreted through these basic data.

Bacteriologic procedures for measuring sanitary ventilation follow the principles laid down by Pettenkofer in determining ventilation load by measuring the equilibrium concentration of carbon dioxide constantly expired per occupant. Lethal equivalents by air disinfection, however, can be determined only by bacteriological methods. Test organisms are atomized at a constant rate into the atmospheres and their rate of elimination determined by the air centrifuge (A.P.H.A., 1938, 1942).

The technique is adapted to the form of the space and the ventilating conditions to be measured. In ordinary rectangular rooms up to 10,000 cubic feet capacity, the following setup should yield satisfactory results under normal winter conditions. An air centrifuge is centrally placed at working level and four atomizers are placed at the same height about half way (about 10 feet) between the centrifuge and corners of the room. An atomizer⁴ containing a liter of water to which has been added 10–15 ml. of a 24-hour culture of *Escherichia coli* in lactose broth, delivers a constant amount (about 1 ml. per minute) over test periods.

The concentration of bacteria in a room usually reaches equilibrium in twenty minutes, depending upon the ventilating rate. Since equilibria are reached more quickly at higher rates of ventilation, it is better to commence a series with maximum ventilating rate and progress to minimum air change. After each equilibrium concentration following a ventilation change has been reached, a series of three 5-minute samples at minute intervals are collected on eosin methylene-blue agar. Atomizers are then turned off and a similar series immediately collected to compute the die-away.

The working rule for obtaining equivalent sanitary ventilation from this

⁴ The "Fragrant Mist" atomizer, manufactured by Walton Laboratories, Inc., Irvington, N. J., meets these specifications.

die-away becomes: *the number of lethoses or overturns per hour is equal to 138 times the difference in the logarithms of two counts divided by the elapsed time in minutes between the two counts.* Equilibrium concentration being proportional to rate of elimination, if rate of addition of infection remains constant, equivalent air change can be readily computed for each ventilating condition.

Concentration gradients. Pollution gradients from infection to disinfection zones (A.P.H.A., 1942) can be studied by simultaneous sampling of a single source of infection at different points. Gradients are inherent in ventilation, and also result from the limited viability of some organisms. They depend upon the relative rates of mixing and disappearance of the organisms, the measurement of which may be required to interpret the pattern of spread of infection among members of an aggregation.

Interpretation. Ultimate interpretation must be based upon broad sanitary principles and epidemiologic experience. Their meaning in terms of air-borne infection and disinfection will become apparent as data accumulated by these procedures are correlated with hygienic indices of the spread of contagion.

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* The disposal of bacteria by air replacement and by disinfection can strictly be equated only for the particular organism tested but the vulnerability of different microorganisms to ultraviolet radiation is sufficiently uniform to justify the interchangeability of these units in ventilating practice.

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GROWTH REQUIREMENTS OF CLOSTRIDIUM TETANI

II. FACTORS EXHAUSTED BY GROWTH OF THE ORGANISM

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Preliminary results of an investigation of the growth requirements of a strain of *Clostridium tetani* have been reported from this laboratory by Mueller and Miller (1942). The information obtained in that study has been applied in attempting to devise a peptone-free medium for the production of tetanus toxin intended for conversion to toxoid for human use. A measure of success has attended this effort, but the level of toxin production achieved has been no better than borderline in terms of the potency required by National Institute of Health regulations, (Mueller and Miller, 1943). It has therefor seemed essential to re-investigate the various factors concerned in growth, and at the same time to check each recognized growth factor against toxin production, in an effort to raise toxin titers three- or four-fold to a concentration comparable with the best yields obtained on peptone-containing media. In this communication, and the one to follow, the results of two separate approaches to the question of growth requirements will be described. The relationship of these findings to toxin production will be presented subsequently.

In the previous work on growth factors, an acid hydrolysate of casein reinforced with tryptophane had been employed as a source of the essential amino acids. This procedure was chosen for two reasons. In the first place, on the basis of much earlier work with various strains of *Corynebacterium diphtheriae* it was shown that the particular amino acids required for prompt and heavy growth varied considerably from one strain to the next. No general conclusions as to the group as a whole could be made from a detailed knowledge of a single strain. It seemed probable that a similar variation would occur with diverse cultures of tetanus. In the second place, as a practical large-scale production method, matters of cost and simplicity made the use of an acid hydrolysate definitely preferable to that of a number of pure amino acids.

There are, however, obvious disadvantages in the use of a relatively crude acid hydrolysate. The amino acids may occur in other than optimal distribution, so that in order to secure an adequate concentration of one, disproportionately large amounts of others will be present, and these may exert an unfavorable influence either through osmotic effects or in other ways. Moreover, when a substance as crude as commercial casein is employed for the preparation of the

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the President and Fellows of Harvard College.

² Aided by a grant from the Commonwealth Fund.

hydrolysate, it is evident that compounds other than amino acids will inevitably be found in the product,—substances which themselves may affect growth and toxin production either favorably or otherwise.

Evidence was at hand which indicated that one or more limiting factors in the hydrolysate medium were responsible for the comparatively low titers of toxin obtained. In the earlier work on diphtheria toxin production it had been shown that toxin production did not cease because the maximum amount compatible with continued growth of the organism had been produced. Rather, growth and toxin production were arrested when some essential component of the medium became used up. By allowing the pellicle of *C. diphtheriae* to form on the surface of a thin layer of fluid medium superimposed on a reservoir of the same medium incorporated in a relatively thick layer of agar, and separated from the fluid phase by a sheet of cellophane, extremely heavy growth and high concentrations of toxin were obtained (Mueller, 1939). The toxin was unable to diffuse away from the fluid whereas any nutrient material which was utilized was promptly replaced from the excess in the agar. Inert ingredients remained equal on both sides of the membrane. A similar experiment was done by Brewer (1941) with *C. tetani*, except that the nature of the growth (absence of pellicle) makes its conduct somewhat simpler. One suspends a loop of cellophane sausage casing in a flask or bottle of the medium, inoculating only the contents of the bag. Here again, and by the same principle, a very considerable concentration of toxin is obtained. This observation has been confirmed in our laboratory.

The identity of the components of the medium which limit growth has therefore been sought, bearing in mind the possibility that there might also be involved the question of a production of inhibitory metabolic products in themselves responsible for the phenomenon. The method has consisted in growing our strain (New York State Laboratories) of *C. tetani* in 4-liter bottles of the hydrolysate medium, prepared as for toxin production, for periods of four to six days, then autoclaving at 15 lbs. to destroy spores and toxin, and to coagulate the greater part of any soluble protein resulting from the metabolism of the organisms. The turbid solution was filtered through paper, and preserved with chloroform in the cold room for use. For experiment, a sufficient amount of such an "exhausted medium" was distributed in 10 ml. amounts in test tubes, suitable additions of test materials were made, the pH adjusted to 7.4–7.6, the mixtures sterilized at 10 lb. for 6–8 minutes, cooled, and inoculated. For this purpose 10 ml. of a 24-hour culture on peptone-infusion broth was centrifuged, the supernatant removed and the sediment suspended in about 1.0 ml. of saline. With a capillary pipette, 0.05–0.1 ml. of the suspension was added to each tube. Incubation was at 34°–36° for 24 hours, and the growth was read in a Gates suspensimeter.

Attention was directed primarily to the casein hydrolysate component of the medium for the reasons outlined above. Preliminary experiments had given evidence of the necessity for histidine, arginine, glutamic acid and tryptophane, as well as for other unidentified amino acids. It was especially in regard to the last named group that it was hoped to obtain information by this method.

As the experiments proceeded it became evident that the most important single

limiting growth factor in the medium as constituted for optimal toxin production was iron. This element, essential for growth of *C. tetani* as for *C. diphtheriae*, prevents in both cases an abundant production of toxin except in extremely low and carefully controlled concentrations. For optimal tetanus toxin, an amount of the order of 0.05 micrograms of Fe per ml. is required. Below this, growth diminishes sharply while with larger quantities growth improves, but toxin diminishes. Exhausted media prepared in this range of Fe could occasionally be reactivated simply by the further addition of Fe salts so as to give a certain amount of growth.

Even at this point, however, it became evident that tryptophane in the amount employed was a second limiting factor. This amino acid had been reduced, because of its expense, to a relatively low concentration, and evidently dangerously near the level of deficiency. When both tryptophane and iron were increased in the initial medium, or when the medium originally prepared as described above was exhausted a second time after the addition of supplements of Fe and tryptophane, a more complex set of deficiencies was uncovered.

In the first place, a definite requirement for biotin appeared. The necessity for this substance had been suggested in earlier work by the inhibition of growth by avidin, and it was assumed to be present in the casein hydrolysate or other components of the medium as an impurity. Such is evidently the case, and under suitable conditions the small amount available from these sources becomes exhausted. Either free biotin or the methyl ester, (both prepared from naturally occurring sources), will supplement such media, the amount required being of the order of 0.001 microgram per ml. or less.

A new and unanticipated growth factor appeared in the form of oleic acid. This substance, shown by Cohen, Snyder and Mueller (1941) to be essential for the growth of *C. diphtheriae* from minute inocula, obviously occurs in casein hydrolysate due to the presence in commercial casein of small amounts of butter fat. Its function as a growth factor for *C. tetani* was demonstrated through the finding that the exhausted medium, reinforced with Fe, tryptophane and biotin was still deficient in a substance present in the "proline" fraction obtained by Dakin's butyl alcohol extraction of casein hydrolysate. The active material could be extracted from this fraction with ether (evidently as the butyl ester), and also it could be extracted from whole commercial casein by ether or alcohol. The knowledge that oleic acid had been found essential under certain circumstances for the diphtheria bacillus quickly led to its successful substitution for the casein factor. Since oleic acid is not available in synthetic form, the possibility remains that another substance present as an impurity, rather than oleic acid itself may be concerned. This possibility had previously been considered in connection with the work on *C. diphtheriae* mentioned above, and had been excluded so far as possible by chemical purification of commercial oleic acid. This was accomplished by converting the material to its methyl ester, which was fractionally distilled. The appropriate fraction of distillate was converted by bromination to dibrom-oleic-acid-ester, which was again purified by fractional distillation, and finally reduced by means of zinc dust and hydrolyzed. The resulting oleic acid retained fully its effect on growth of *C.*

diphtheriae. A specimen of the same preparation was quite as effective as commercial oleic acid in the present experiments with *C. tetani*.

Folic acid also may become a limiting factor under suitable conditions—(either two exhaustions or adequate Fe and tryptophane in the initial formula). The addition to such media of 0.005 micrograms per ml. of a concentrate of folic acid kindly supplied by Prof. R. J. Williams renders the medium again suitable for use, in the presence of the other factors previously mentioned.

Of the amino acids other than tryptophane, only glutamic acid and histidine appear to be used to the point where their concentrations may become limiting under the experimental conditions here employed. There has appeared to be nothing to be gained through carrying the exhaustion experiments further, for obviously such results could have no bearing on the immediate problem of limitation of toxin production.

It does not seem worth while presenting detailed protocols of experiments by which the above points have been established. In the nature of the case, no two lots of the exhausted base are completely identical, and each batch has had to be treated somewhat as an individual problem,—its particular deficiencies determined and eventually replaced by known compounds and more or less well defined concentrates. It may be said, however, that in the case of each of the substances discussed above, it has been possible to carry through one or more experiments in which little or no growth occurred in the absence of the compound in question, while its addition in suitable quantity resulted in growth approximating that obtained on a peptone-infusion broth.

SUMMARY

Growth of *Clostridium tetani* on a casein hydrolysate medium reinforced with various growth accessories and certain inorganic ions eventually ceases because of the depletion of the medium in respect to one or more nutrilites. Under conditions of maximal toxin production, the iron seems to be the first one to disappear, being presumably linked in organic combination in the cells. In the presence of somewhat more iron, the following substances disappear more or less in the order named: Tryptophane, biotin, oleic acid, folic acid, histidine and glutamic acid.

It appears that iron is the first substance which sharply limits growth as well as toxin and that little is therefore to be expected through the addition of larger quantities of other growth factors to the medium. The actual application of these results to toxin formation will be described later.

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GROWTH REQUIREMENTS OF CLOSTRIDIUM TETANI

III. A "SYNTHETIC" MEDIUM

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In the preceding communication (Feeney, Mueller and Miller, 1943) the relationship of our present knowledge of the growth requirements of *Clostridium tetani* was briefly reviewed in connection with the question of toxin production on synthetic media. Experiments were detailed regarding the nature of the components of the medium which limited growth (and presumably toxin formation). In these experiments definite requirements for biotin and for oleic acid were demonstrated, and it was shown that certain other components of the medium, notably Fe, tryptophane and folic acid were rapidly exhausted by growth of the organism. The present paper presents the results of a parallel series of experiments through which it was sought to identify all of the factors essential to growth. Beginning with a basic formula established as the result of earlier work (Mueller and Miller, 1942) and of unpublished experiments, incapable in itself of supporting growth without the use of supplementary mixtures of partially unknown composition, it was sought to identify the components of the supplements which induced growth. The ultimate objective was a satisfactory medium containing only materials of known composition. To a reasonable extent, this has now been accomplished and the results are here presented. A consideration of the application of the findings to the question of toxin production will for the present be reserved.

EXPERIMENTAL

Preparation of the stock basal media. Since the basal media varied as the experimental work progressed, only the formulae of the initial liver-containing medium and the final synthetic medium will be presented. Modifications of the liver medium leading to the identification of the various additional substances will be indicated in the discussion of these materials. Each preparation of basal medium was made up in amounts sufficient for 50–200 10 ml. test lots. The ingredients other than the materials under investigation were dissolved in a small amount of water containing excess hydrochloric acid. The amount of acid added was varied to give an approximate total concentration of 0.6%–0.8% sodium chloride in the completed test medium after neutralizing with sodium hydroxide. Phenol-red indicator was added and the medium diluted to a volume

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one-fourth of that in which the bacteriological tests were conducted. Basal media so prepared were stored in the refrigerator for over a month with no apparent changes.

The amounts of the constituents of the media as listed in the tables or described in the text are given in terms of the media as diluted for a test run (one-fourth the concentration in the stored stock basal media).

Bacteriological techniques, etc. For each set of determinations portions of the basal media were diluted with water, adjusted to pH 7.4–7.6 with sodium hydroxide, and an amount equivalent to 2.5 ml. of the undiluted basal medium added to 150 x 15 mm. Pyrex test tubes which were previously cleaned by standing overnight in strong chromic-sulfuric acid, thoroughly rinsed with water and dried. The solutions of materials to be tested were added to each tube, any changes in acidity rectified, and the contents of each tube diluted to 10 ml. The tubes were covered with glass caps, autoclaved at 10 lbs. steam pressure for five minutes, removed promptly from the autoclave, cooled in water, and inoculated. The glass caps were replaced by previously sterilized cotton plugs and the tubes were immediately placed in an anaerobic jar (Mueller and Miller, 1941).

The strain of the organism employed and the procedure of carrying the stock culture by serial transfers in tubes of glucose-peptone-infusion broth were as previously described (Mueller and Miller, 1942).

A large inoculum was employed to facilitate rapid growth. During the introductory stages of the problem the cells of one 10 ml. broth culture were centrifuged, resuspended in sterile saline, and used to inoculate 15–20 test lots of media. This was later slightly refined by washing the cells once with 10 ml. of sterile saline and roughly standardizing the amount of inoculum so that one broth culture served to inoculate 25 test lots of media. The inocula were introduced into the bottom of the tubes by means of sterile Pasteur pipettes.

The extent of growth was usually determined after 24–30 hours of incubation at 37° by reading the turbidity with a Gates suspensiometer and comparing the readings with those of serially diluted glucose-peptone-infusion broth cultures. Longer incubation times and simple visual comparisons of turbidities were occasionally employed.

Protein hydrolysates. The casein and gelatin were hydrolyzed with sulfuric acid and the other proteins with hydrochloric acid. The latter hydrolysates were prepared by refluxing the proteins in 8 N acid for 16 hours and removing the excess of the hydrogen chloride *in vacuo*. The sulfuric acid hydrolysates and the butyl alcohol fractions therefrom (the diamino-dicarboxylic, the proline and the monoamino-monocarboxylic acid fractions) were prepared in the usual way.

The liver-extract basal medium. It was found possible to obtain moderate growth in the medium shown in table 1. The evidence for the inclusion of serine, methionine, tyrosine and aspartic acid was based on small increases in growth or toxin production, and in the case of serine and aspartic acid, on the metabolism experiments of Clifton (1942). Nicotinic acid, pimelic acid, beta-alanine, oleic acid and other of the ingredients might well have been omitted in the presence of the liver extract.

dl-isoleucine. The addition of either acid-hydrolyzed casein or gelatin to the liver extract basal medium increased the growth to the level of that obtained in peptone-infusion broth. The amino acid *dl-isoleucine* largely replaced the protein hydrolysates. *Dl-leucine* had a small stimulatory effect.

Valine. The basal medium was now modified by the omission of the liver extract and the inclusion of the following materials per 100 ml. of test medium: 30 mg. *dl-isoleucine*, 30 mg. *dl-leucine*, 500 gamma adenine, 10 gamma riboflavin, and 10 gamma thiamine. The latter three substances were known to be essential in the absence of the liver extract (Mueller and Miller, 1942). No growth occurred in this medium. The addition of hydrolyzed casein resulted in excellent growth and the addition of hydrolyzed gelatin in poor growth. The Dakin diamino-dicarboxylic acid fraction of the gelatin hydrolysate, however,

TABLE 1
Composition of initial basal medium containing liver extract

SUBSTANCE	AMOUNT PER 100 ML.	SUBSTANCE	AMOUNT PER 100 ML.
l-Glutamic acid	400 mg.	Biotin	0.10 gamma
l-Arginine.....	60 mg.	Pantothenic acid	25 gamma
l-Histidine	50 mg.	Folic acid concentrate*.....	0.50 gamma
l-Tyrosine.....	30 mg.	Na ₂ HPO ₄	100 mg.
dl-Serine.....	20 mg.	KH ₂ PO ₄	30 mg.
dl-Methionine	40 mg.	Accessories and metals solution†.....	0.20 ml.
l-Aspartic acid	40 mg.	Liver extract‡	0.10 ml.
l-Cystine.....	40 mg.	Conc. hydrochloric acid.....	0.8 ml.
l-Tryptophane.....	5.0 mg.	FeSO ₄ ·7H ₂ O	100 gamma
Glucose (reagent).....	1.0 g.		
Oleic acid.....	100 gamma		

* The concentrate of folic acid was kindly supplied by Dr. R. J. Williams. It was labeled "Potency 38,000."

† A solution with the following composition per 100 ml. MgSO₄·7H₂O 22.5 g., CuSO₄·5H₂O 50 mg., ZnSO₄·7H₂O 40 mg., MnCl₂·4H₂O 15 mg., beta-alanine 115 mg., nicotinic acid 115 mg., pimelic acid 7.5 mg., conc. hydrochloric acid 1.5 ml.

‡ A 90% ethanol filtrate fraction of a water extract of liver. This fraction was obtained through the courtesy of the Connaught Laboratories, Toronto, Canada.

was almost devoid of the activity found in the whole hydrolysate. Supplementation of the Dakin fraction with *dl-valine* largely restored the activity of this fraction to that of the whole hydrolysate.

Nicotinic acid. The basal medium was further modified by the inclusion of 30 mg. of *dl-valine* per 100 ml. of test medium and the omission of the accessories (pimelic acid, beta-alanine, and nicotinic acid). Likewise, the techniques of washing and standardizing the inoculum were initiated.

Again no growth was obtained in the modified medium. Gelatin hydrolysate was completely inactive while casein hydrolysate supported good growth as before. A hydrolysate of a sample of purified casein was much less effective. Purification of the casein was accomplished by reprecipitation and prolonged extraction with 95% ethanol in a Soxhlet apparatus. The excellent growth pos-

sible with the unpurified casein was again obtained when the hydrolysate of the purified casein was supplemented with nicotinic acid and pyridoxine. The effect of pyridoxine was variable and slight.

Manganese and various amino acids. With the inclusion of nicotinic acid and pyridoxine at a level of 100 gamma of each per 100 ml. of test medium, the study of the unknown materials supplied by the casein was continued. Testing of the three Dakin fractions, the diamino-dicarboxylic acid, the proline, and the mono-amino-monocarboxylic acid, revealed that both the diamino-dicarboxylic acid fraction and the proline fraction were required for growth. This separation was not entirely complete as the diamino-dicarboxylic acid fraction was sufficient by itself at high levels. Since the proline fraction could not be replaced by mixtures of amino acids or other protein hydrolysates, its investigation was temporarily deferred and it was included in the test medium at a level equivalent to 500 mg. of casein per 100 ml. of test medium and the materials in the other fraction studied.

The diamino-dicarboxylic acid fraction was completely replaced by acid hydrolysates of gelatin, edestin, pumpkin seed globulin and largely by an acid hydrolysate of hemoglobin. A partial replacement of this fraction was achieved by adding a mixture of 10 mg. per 100 ml. of medium of each of the following amino acids: lysine, oxyproline, proline, threonine, glycine, alanine, and phenyl-alanine. The effect of this mixture was extremely inconstant, varying from slight to good growth from test to test and with successive batches of media. This variation was completely removed and good growth was consistently obtained by the further addition of an ash of casein. A fairly large number of metallic salts were tested and, of these, manganese chloride tetrahydrate was found to replace the ash at a level of 100-200 gamma per 100 ml. of medium. The other salts tested were without effect.

Uracil. When the above mixture of amino acids and more manganese were added to the medium and the proline fraction of casein was omitted, no growth occurred. Of a number of materials tested, a 90% ethanol soluble fraction of an aqueous extract of liver appeared the most feasible source for the study of the properties of the unknown active substance.

The activity of the liver extract was not diminished by prolonged acid hydrolysis or by treatment with nitrous acid. It was largely absorbed on Norit from acid solution and eluted from the Norit by boiling 50% ethanol. Extraction experiments with diethyl ether and butanol demonstrated that the active material extracted slowly with ether and rapidly with butanol from strongly acid solution (10% sulphuric acid) but would not extract from strongly alkaline solution. After saturation of the alkaline solution with carbon dioxide, the activity was again extractible.

A concentrate of the active material was easily prepared by utilizing the above described properties. The liver extract was absorbed on and eluted from Norit and the eluate extracted from strongly acid solution with ether in a continuous extraction apparatus. The easily water-soluble solids of the ether extract were dissolved in water and the water solution extracted several times with an equal

volume of ether. The water solution was then made strongly alkaline with sodium hydroxide, extracted 15 times with a double volume of butanol, and finally 15 times with a double volume of butanol after saturation with carbon dioxide. The concentrate obtained in the final butanol extract contained over two-thirds of the total activity of the original liver extract and gave a maximum growth response at a level of 150–200 gamma per 100 ml. of test medium.

At this point it was found that the majority of the activity was precipitated by silver nitrate and barium hydroxide, and several purines and pyrimidines were tested. The pyrimidine uracil completely replaced the concentrate while thymine, guanine, and hypoxanthine were without effect. Uracil was included in the medium at a level of 250 gamma per 100 ml. of medium.

TABLE 2
Composition of the synthetic medium

SUBSTANCE	AMOUNT PER 100 ML.	SUBSTANCE	AMOUNT PER 100 ML.
d-Glutamic acid..	250 mg.	Biotin.....	0.10 gamma
l-Arginine	50 mg.	Pantothenic acid.....	25 gamma
l-Histidine	50 mg.	Folic acid concentrate*.....	0.50 gamma
l-Tyrosine	30 mg.	Na ₂ HPO ₄	100 mg.
dl-Serine	20 mg.	KH ₂ PO ₄	30 mg.
dl-Methionine.....	20 mg.	Metals†.....	0.20 ml.
dl-Aspartic acid.....	20 mg.	FeSO ₄ ·7H ₂ O... ..	100 gamma
l-Cystine	40 mg.	HCl.....	0.80 ml.
l-Tryptophane	5.0 mg.	Glucose	1.0 gram
dl-Valine	30 mg.	Riboflavin.....	10 gamma
dl-Isoleucine	30 mg.	Thiamin.....	10 gamma
dl-Leucine	30 mg.	Nicotinic acid.....	100 gamma
dl-Threonine	20 mg.	Pyridoxine.....	100 gamma
dl-Phenylalanine	20 mg.	Uracil.....	250 gamma
dl-Lysine	20 mg.	Adenine.....	500 gamma
Oleic acid	250 gamma		

* See table 1.

† As in table 1—without accessories.

Pyridoxine. Since pyridoxine had originally been added to the medium with little basis for its use, the effect of its omission was now studied. In primary culture little effect was noted, but upon subculturing the organism in the medium it proved essential. Though the growth with pyridoxine was slower and a little less heavy in subculture, no growth was obtained in its absence. Pyridoxine was therefore retained in the medium.

The final constitution of the synthetic medium and requirements in this medium. Various of the amino acids which had previously been incorporated in the medium were now found to have no influence on growth and were omitted. The final constitution of the synthetic medium is listed in table 2. Though the growth in this medium was consistently good, it was slower and a trifle less heavy than in glucose-peptone-infusion broth. The growth obtained after 24–30 hours of incubation was usually 80–85% of that obtainable in broth.

When the amino acids of the optimum mixture were omitted from the medium one at a time (with the exception of tryptophane and cystine), varying effects on growth were observed (table 3). Tyrosine, leucine, isoleucine, histidine, valine and arginine were essential for growth. Threonine, serine and phenylalanine were strongly stimulatory, while methionine, lysine and aspartic acid appeared only slightly stimulatory. Glutamic acid was not essential and had little effect on the stimulation of more rapid growth but permitted much heavier growth. Synthetic dl-glutamic acid replaced the natural amino acid. Although no attempt was made to study more extensively the action of glutamic acid and the possibility of its replacement by other substances, it was considered as serving as a source of energy for the organism. The minimum requirement of each of the

TABLE 3
*Growth of organism in absence of individual amino acids**

AMINO ACID OMITTED	RELATIVE GROWTH AFTER INCUBATION FOR†		
	24 hours	48 hours	72 hours
None.....	++++	++++	++++
d-Glutamic acid.....	++	++	++
l-Tyrosine.....	—	tr	tr
dl-Leucine.....	—	—	—
dl-Isoleucine.....	—	—	—
l-Histidine.....	—	—	—
dl-Valine.....	—	—	—
l-Arginine.....	tr	—	—
dl-Serine.....	tr	++	++++
dl-Phenylalanine.....	++	+++	++++
dl-Threonine.....	tr	++	++++
dl-Lysine.....	+++	++++	++++
dl-Methionine.....	+++	++++	++++
l-Aspartic acid.....	++	++++	++++
All thirteen above.....	—	—	—

* In synthetic medium.

† Relative growth estimated by visual observation. — = none, tr = barely visible turbidity, + to ++++ gradations to maximum.

essential amino acids (except tryptophane) was between 100 and 300 gamma per 10 ml. of test medium. The optimum amount of glutamic acid was between 15 and 25 mg. per 10 ml. of test medium.

Oleic acid, biotin, nicotinic acid, uracil, and pyridixone were all essential. No growth was obtained after 24–30 hours of incubation when any one of these substances was omitted from the medium. After three or four days of incubation only the cultures from which uracil had been omitted had any visible growth. This growth was 15–20% of the growth obtained in 24–30 hours in the presence of uracil and did not increase with still further incubation. In table 4 are listed the responses of the organism to graded amounts of these substances. The minimum requirements per 10 ml. of medium for each of these materials were as follows: nicotinic acid 0.50 gamma, pyridoxine 0.5 gamma, biotin 0.0075 gamma,

uracil 5.0 gamma and oleic acid 7.5–10.0 gamma. Nicotinamide replaced nicotinic acid microgram for microgram. The above experiments concerning pyridoxine were performed in subculture.

TABLE 4
Response of organism to graded amounts of nicotinic acid, uracil, biotin, oleic acid, and pyridoxine

MATERIAL		RELATIVE GROWTH†
Omitted*	Amount added	
	<i>per 10 ml.</i>	<i>per cent</i>
Oleic acid	10.0 gamma	100
	7.5 gamma	100
	5.0 gamma	55
	2.5 gamma	25
	none	0
Uracil	10.0 gamma	100
	5.0 gamma	100
	2.5 gamma	65
	1.0 gamma	45
	0.5 gamma	25
	none	0
Biotin	0.0100 gamma	100
	0.0075 gamma	90
	0.0050 gamma	50
	0.0025 gamma	10
	none	0
Nicotinic acid	0.100 gamma	100
	0.050 gamma	100
	0.025 gamma	70
	0.010 gamma	55
	0.005 gamma	35
	none	0
Pyridoxine‡	10.0 gamma	90
	1.0 gamma	90
	0.2 gamma	45
	none	0

* From synthetic medium.

† The maximum growth under these conditions was approximately 80% of that obtainable in broth. The relative growths are expressed in percentages of this maximum growth.

‡ The experiments on pyridoxine were performed in first subculture after 36 hours of incubation.

DISCUSSION

Although good growth of *Clostridium tetani* occurred in the synthetic medium, unknown stimulatory substances still exist and it is possible that unknown essential substances also exist. No attempt was made to carry the organism further

than the first subculture and even the growth in the first subculture was not optimum. Serial subcultures with a small inoculum might well reveal a further deficiency of an essential substance. One such substance might be the sporogenes vitamin which has been shown to be required by various pathogenic clostridia (Fildes, 1935). None of the materials found essential for the growth of this strain of the tetanus bacillus appears to have the chemical properties ascribed to the sporogenes vitamin (Knight and Fildes, 1933) (Pappenheimer, 1935). The natural amino acids, glucose or the folic acid concentrate may possibly have contained essential impurities.

Beyond the demonstrated requirements for K, Mg, PO₄, Fe and Mn, nothing is known of the mineral requirements of the organism. The copper and zinc salts included in the medium have been omitted without any effect on growth.

An interesting observation in the course of the problem was the apparent presence of the amino acid valine in the gelatin hydrolysate, as judged from the growth response of the organism. A sample of the same gelatin hydrolysate was found to support the growth of *Lactobacillus arabinosa* when added to a medium containing no valine.³ Since this organism has been found to require valine for growth (Hegsted, 1943) it would appear that our observation has been substantiated, and that gelatin contains a small amount of valine. This is contrary to accepted analytical data, (Dakin, 1920).

Uracil was not isolated from the concentrate and only a very few materials were tested in its place, but it seemed probable that the active material in the liver extract was uracil. It was well defined as a weakly acidic substance without a basic functional group. The concentrate was approximately one-fourth as active as uracil on a weight basis.

An extensive report of the requirements of the organism for riboflavin, thiamin, folic acid, pantothenic acid, adenine and tryptophane in a well-defined medium has been published (Mueller and Miller, 1942) and protocols concerning these materials have been presented. Both glucose and cystine were included in the medium, though either one of these alone was previously reported as being sufficient. Oleic acid, biotin, histidine, and glutamic acid were reconsidered as the original investigation concerning their requirement was conducted in a medium of unknown composition (Feeney, Mueller and Miller, 1943).

Little information concerning the nutrition of the other pathogenic clostridia has been published to date. Fildes and Richardson (1935) reported that leucine, arginine, tyrosine, phenylalanine, and tryptophane were essential and valine, histidine, cystine, and methionine were stimulatory and possibly essential for the growth of *Clostridium sporogenes*. Following the discovery of oleic acid as an essential factor for *Clostridium tetani*, Dr. A. M. Pappenheimer, Jr., has found *Clostridium welchii* to require oleic acid, pyridoxine, and uracil.⁴ It appears possible that the majority of the pathogenic clostridia will be found to have as complex nutritional requirements as is the case with *C. tetani*.

³ The authors are grateful to Dr. D. M. Hegsted for this determination.

⁴ The authors are indebted to Dr. A. M. Pappenheimer, Jr., for suggesting that uracil might be required by *Clostridium tetani*.

Since the fundamental purpose of these investigations has concerned the production of tetanus toxoid for the armed forces, various interesting but academic phases of the problem have been neglected for the present. Further experiments on the nutrition of the organism will be conducted only insofar as studies on toxin production indicate their importance. Toxin studies with a medium similar to that reported here are encouraging at the present time.

SUMMARY

1. Good growth of a strain of *Clostridium tetani* has been obtained in a synthetic medium. Initial subculture was possible but was not entirely satisfactory.

2. In addition to the following previously reported essential substances: riboflavin, pantothenic acid, thiamin, folic acid, adenine, biotin, and oleic acid, the following materials have been found essential: uracil, pyridoxine, and nicotinic acid.

3. The following amino acids were essential: arginine, histidine, tyrosine, valine, isoleucine, leucine, and tryptophane.

4. The amino acids, threonine, phenylalanine, and serine, greatly stimulated the rate of growth, while lysine, aspartic acid and methionine stimulated to a small extent. Glutamic acid supported heavy growth but was not essential.

5. Evidence was obtained that manganese is necessary for optimal growth.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

CENTRAL NEW YORK BRANCH

AGRICULTURAL EXPERIMENT STATION, GENEVA, N. Y., OCTOBER 23, 1943

ABSENCE OF d-AMINO ACIDS IN THE PROTEINS OF VIRUS-INDUCED TUMORS IN RABBITS. *Henry W. Scherp*, University of Rochester, Rochester.

ORGANIC ACIDS AS SUBSTRATES FOR STREPTOCOCCI. *J. J. R. Campbell, W. D. Bellamy, and I. C. Gunsalus*, College of Agriculture, Cornell University, Ithaca.

Though certain potentialities of an organism may be studied by varying the growth conditions with a given substrate, others may best be studied by a change in the type of substrate. The reactions of lactic acid bacteria toward most organic acids seem to fall in the latter group.

Streptococcus fecalis (10 Cl) was grown in a neutral medium with various organic acids as energy sources and the growth compared to that obtained with glucose. On a molar basis, where glucose = 100, citric acid = 35, malic 20, oxalacetic 15, pyruvic 5.

The activity of cell suspensions harvested after growth on these energy sources has been tested in the Warburg apparatus. Glucose-grown cells were not active in the fermentation or oxidation of malic and citric acids. Malic- and citric-grown cells were 10 to 100 times more active in oxidation of their respective substrates than were other cells. Cells adapted to these acids fermented glucose more slowly than glucose-grown cells, but citric acid cells oxidized glucose more rapidly and with greater oxygen utilization than glucose-grown cells. Pyruvic acid also was oxidized more rapidly by citric-grown cells. The relation of these factors to the lactic acid fermentation was discussed.

GROWTH REQUIREMENTS OF STREPTOCOCCUS FECALIS FOR TYROSINE DECARBOXYLATION. *W. D. Bellamy and I. C. Gunsalus*, College of Agriculture, Cornell University, Ithaca.

The effect of the growth medium upon the tyrosine decarboxylation activity of resting

cells of *Streptococcus fecalis* (10 Cl) has been studied. Since Gale reported the optimum pH for the production and action of tyrosine decarboxylation to be 5, the activity of cell suspensions was tested in phthalate buffer, pH 5, using a Warburg apparatus. Rapid decarboxylation was obtained with cells from a medium containing 1 per cent tryptone, 1 per cent yeast extract, 0.5 per cent glucose and 0.5 per cent K_2HPO_4 . The tryptone could be replaced by acid-hydrolyzed casein, fortified with cystine and tryptophane, without decrease in activity or growth. Acid-hydrolyzed gelatin was equivalent to casein in the medium for growth but yielded inactive cells unless supplemented with tyrosine.

When the tryptone was replaced by hydrolyzed gelatin, supplemented as above, a decrease in yeast extract from 1 to 0.2, per cent caused a marked decrease in the decarboxylation activity. The addition of pyridoxine and nicotinic acid restored the activity without materially affecting growth. Replacement of the yeast extract by accessory factors and salts yielded reasonably good growth. For rapid tyrosine decarboxylation, however, the pyridoxine and nicotinic acid were required in 5 to 10 times the concentration necessary for maximum growth.

THE NUTRITION OF GROUP B STREPTOCOCCI.

C. F. Niven, Jr., College of Agriculture, Cornell University, Ithaca.

The nutritive requirements of 18 strains of Group B streptococci isolated from raw milk and human sources have been investigated. Using a basal medium containing hydrolyzed casein, tryptophane, phosphate buffer, glucose, sodium thioglycolate and inorganic salts, all strains tested required biotin, nicotinic acid, pantothenic acid, thiamine, and pyridoxine for growth. Sixteen strains were able to grow in the absence of riboflavin.

An additional unknown growth factor is

probably required by 8 strains as evidenced by their inability to survive repeated transfers in the simplified medium when small inoculums were used. Glutamine, asparagine, p-aminobenzoic acid, choline, inositol, and folic acid concentrate, added singly or in combination, did not alter the growth of these strains.

A mixture of 20 amino acids successfully replaced the casein hydrolysate for the 18 cultures tested. When omitted singly from the complete mixture, valine, leucine, isoleucine, phenylalanine, glutamic acid, arginine, lysine, histidine, and tryptophane were found to be essential for the two strains tested; while threonine, cystine, methionine and tyrosine stimulated growth. No significant differences were found in the nutritive requirements of the strains isolated from milk and from human sources.

TAXONOMY OF CERTAIN MEMBERS OF THE GENUS *CORYNEBACTERIUM* FROM ANIMAL SOURCES. *Robert F. Brooks*, N. Y. State Agricultural Experiment Station, Geneva.

A recent study of 79 stock and recently isolated strains of the genus *Corynebacterium* secured from normal and pathologic animal sources has thrown some doubt on the usefulness of the present arrangement of species in this genus, which is based largely on habitat relationships. Several characters heretofore thought to be of primary value in differentiating the members of the genus have shown a wide range of variability.

An attempt to correlate the characteristics of these 79 cultures so as to arrive at a valid system of species differentiation

proved to be practically futile. For this reason, and because of the variability of cultures previously classified as belonging to the same species, it is felt that the only valid arrangement of the cultures studied is one which establishes three rather broad groups, which agree in regard to the following six characters:

1. Presence or absence of a pellicle in nutrient broth.
2. Final reaction in litmus milk.
3. Pigment production on coagulated blood serum.
4. Reduction of nitrates to nitrites.
5. Production of hydrogen sulfide.
6. Final reaction in glucose nutrient broth.

A further, more complete report will deal with the arrangement and characteristics of these three groups in more detail.

THE GRAM-POSITIVE PHYTOPATHOGENIC BACTERIA. *W. H. Burkholder*, Department of Plant Pathology, College of Agriculture, Cornell University, Ithaca.

THE EFFECT OF FEVER ON THE DISTRIBUTION OF ARSENIC IN RABBITS FOLLOWING THE INTRAVENOUS ADMINISTRATION OF MAPHARSEN. *H. E. Stokinger, F. L. Dorn, R. A. Boak, and C. M. Carpenter*, University of Rochester, Rochester.

TYPES OF FLAGELLATION FOUND AMONG BACTERIA. *Robert S. Breed*, N. Y. State Agricultural Experiment Station, Geneva.

FLAGELLATION OF AZOTOBACTER. *Alvin W. Hofer*, N. Y. State Agricultural Experiment Station, Geneva.

INDIANA BRANCH

**JOINT MEETING WITH BACTERIOLOGY SECTION, INDIANA ACADEMY OF SCIENCE
BUTLER UNIVERSITY, INDIANAPOLIS, OCTOBER 29, 1943**

A COMPARISON OF THE ACTION OF SULFADRUGS ON THE GROWTH OF A BACTERIAL VIRUS AND OF ITS HOST. *M. Delbrück and S. E. Luria*, Department of Physics, Vanderbilt University, Nashville, Tennessee, and Bacteriological Laboratories, Indiana University, Bloomington, Indiana.

These experiments were performed to attempt to dissociate the growth of bacterial

viruses from the growth of their host by blocking certain enzymes of the bacterial cell. The growth of both host and virus in a synthetic medium was quantitatively studied, and the action of sulfathiazole investigated. As shown by previous authors, ST(10^{-4} - 10^{-5} M) reduces the bacterial growth rate after a latent period of a few hours. Whether the growth continues at this reduced rate or is replaced after a few

hours by rapid death of bacteria depends on the size of the initial inoculum. PAB inhibits the action of ST; the antagonism is strictly quantitative. Normal growth takes place with ratios of PAB/ST higher than 1/7. The experiments on the effect of ST on virus growth, as yet incomplete, show a strict correlation between inhibition of bacterial growth and of virus growth; the latter is reduced in the same proportion as is the growth rate of bacteria. The reduction in virus growth takes place only when the host has been grown in presence of ST long enough to show an appreciable reduction of its growth rate. Possible interpretations of these results in connection with the rôle of bacterial enzymes in the growth of virus are suggested.

DISSOCIATION OF THE GROWTH OF BACTERIAL VIRUSES AND OF THEIR HOST BY MEANS OF TEMPERATURES ABOVE OPTIMUM.¹ *S. E. Luria*, Bacteriological Laboratories, Indiana University, Bloomington, Indiana.

For two coli-viruses, temperatures between 15° and 40°C. affect the growth of both host and virus in a strictly parallel manner. At temperatures of 43° and 45°C. the bacteria can grow at reduced rates; if, however, bacteria infected at 37°C. are transferred to 43° or 45°C., no liberation of virus takes place. If the bacteria are then returned to 37°C., virus is liberated, in small amount, and after a delay increasing with increased stay at high temperature. These results seem to suggest that one or more of the reactions involved in the production of new virus are inhibited at or above 43°C. The inhibition must take place through a reaction with very high temperature coefficient. Some essential reagent is probably removed or inactivated, as indicated by the delay and incompleteness of the restoration of virus production after return to normal temperature. There is as yet no indication as to whether the inhibition concerns the actual multiplication of the virus in the host or only its liberation from the host (lysis).

¹ Experiments done in the Department of Biology, Princeton University, under the tenure of a Guggenheim Fellowship.

EFFECT OF VARIOUS CONCENTRATIONS OF IRON ON THE PRODUCTION OF RIBOFLAVIN BY CERTAIN CLOSTRIDIA. *Allen Saunders and L. S. McClung*, Bacteriological Laboratories, Indiana University, Bloomington, Indiana.

In a study of riboflavin production by various aerobic and anaerobic bacteria, Rodgers (1942) reported that fortification of the iron content of the corn mash used for fermentation by *Clostridium acetobutylicum* increased the production of riboflavin. Confirmation of this stimulatory effect of iron has been noted in the case of 4 out of 5 strains of this species in corn mash fermentations to which Fe as FeSO₄ was added in 2, 4, and 6 × 10⁻⁴M concentrations. The effect appears restricted to *C. acetobutylicum*, however, as *C. roseum*, *C. felsineum*, and other pigmented anaerobes including certain yellow butyric types do not respond to the addition of iron.

NATURAL BACTERICIDINS IN THE PLASMA OF THE DOMESTIC FOWL. *E. E. Schnetzler*, Purdue University Agricultural Experiment Station.

The plasma of the domestic fowl contains natural bactericidins capable of killing *Salmonella pullorum* organisms. Wide differences in resistance to the bactericidal action were found between the eight strains of *S. pullorum* employed. Those strains that had been isolated most recently were more resistant to bactericidal action. However, there was considerable variation between the strains within this group.

The plasma of White Leghorns of two different strains showed higher bactericidal activity than that of Rhode Island Reds and White Rocks. The greater bactericidal action of the plasma of White Leghorns may partially account for less infection being observed in this breed.

Wide differences in bactericidal activity have been found between fowls of a given strain. These wide differences observed among stock reared in the same flock indicate genetic differences. After three generations of selection two lines of Rhode Island Reds have been produced differing in bactericidal activity. The results obtained indicate that the bactericidal action

is in part at least influenced by heritable factors.

The bactericidal action of the plasma apparently involved a natural antibody and complement. The plasma of fowls showing low bactericidal activity was apparently not deficient in complement.

A TECHNIQUE FOR THE PRODUCTION OF ANTISERA FOR *PARAMECIUM AURELIA*. *L. S. McClung*, Bacteriological Laboratories, Indiana University, Bloomington, Indiana.

A new technique is described for the production of antisera for *Paramecium aurelia*. This consists of the injection into the marginal ear veins of normal rabbits of the supernatant from a centrifuged suspension of the cells of this organism. The cell suspension is subjected to mechanical agitation before the centrifuging. The serum thus obtained when mixed with living *P. aurelia* cells, causes an immobilization reaction. It is unnecessary to use paramecia which are free from bacteria as the antigen for the serum produced by this technique shows no reaction with the *Aerobacter aerogenes* culture used for feeding the paramecium.

APPLICATION OF THE ELECTRON MICROSCOPE TO BIOLOGICAL RESEARCH. *Lucile J. Weiss*, Lilly Research Laboratories, Indianapolis, Indiana.

The parallelism of light to electron optics and its application to the electron microscope are discussed. The machine is described as to appearance, magnification, photographic ability; and a method is given for preparation of specimens, and their appearance under the microscope described.

The electron microscope as used in biological research has three definite practical applications: First, the observation of organisms under different circumstances, such as, bacteria grown on various suitable media, or the comparison of normal to medicated bacteria. Second, there may be a routine study of vaccines to find possible small variations. Third, there is pure research in the field of organisms not well known or too small to be studied completely with the facilities offered by the light microscope, such as, rickettsiae or virus forms like pleuropneumonia.

BACTERIOLOGY AT BUTLER UNIVERSITY. *C. M. Palmer*, Department of Botany, Butler University, Indianapolis, Indiana.

A course in general bacteriology was organized about twenty years ago and has been offered every year since that time. It is now supplemented by courses in Applied Microscopy and Phycology. Unique features of the course include provisions for each student to do all of his laboratory work from preparation and sterilization of media to washing of glassware, and a comprehensive exercise near the end of the course in the identification of bacteria. A laboratory manual, written especially for the course at Butler University, is now in its fourth edition.

HISTORY OF BACTERIOLOGY AT DEPAUW UNIVERSITY. *T. G. Yunker*, Department of Botany, DePauw University, Greencastle, Indiana.

A brief historical account concerning the introduction of bacteriology in the curriculum at DePauw University.

NOTES CONCERNING THE HISTORY OF BACTERIOLOGY AT INDIANA UNIVERSITY. *L. S. McClung*, Bacteriological Laboratories, Indiana University, Bloomington, Indiana.

Bacteriology was first offered at Indiana University in September, 1896, as an evening course taught by Professor Robert E. Lyons, in the Department of Chemistry. A series of courses were developed by him and were continued until about the time of the establishment of the School of Medicine in 1903. Shortly after this date the work in bacteriology was transferred to the Medical School; and, soon, the Bloomington Campus was without instruction in bacteriology, as the work of the last three years of the School of Medicine was transferred to Indianapolis. This continued until the fall of 1940 when bacteriology was reintroduced into the curriculum of the College of Arts and Sciences by the author.

HISTORY OF BACTERIOLOGY IN INDIANA: PURDUE UNIVERSITY. *P. A. Tetrault*, Purdue University, Lafayette, Indiana.
The history of bacteriology at Purdue University from its beginning to date is

recorded with a brief sketch of the individuals who have helped to develop the science.

THE HISTORY OF BACTERIOLOGY AT THE UNIVERSITY OF NOTRE DAME. *James A. Reyniers and Robert F. Ervin*, Laboratories of Bacteriology, University of Notre Dame, Notre Dame, Indiana.

Bacteriology has been taught at Notre Dame for some 55 years. University catalogues and bulletins indicate that Rev. Alexander M. Kirsch, C.S.C. and later Rev. George Albertson, C.S.C. taught most of the courses in bacteriology between 1890 and 1929. It is possible, but not recorded, that Dr. John Berteling, an M.D., taught the course as early as 1888. Professor James A. Reyniers has taught bacteriology and related courses since 1931. Under the direction of Reyniers, the Laboratories of Bacteriology have been developed primarily for carrying out a research program more or less independent of the academic organization of the University. The first phase of this program, *i.e.*, the perfection of techniques, was almost completed when the advent of war required full attention to more immediate problems.

EPIDEMIC INFLUENZA VACCINE AND ANTISERUM. *H. M. Powell*, Lilly Research Laboratories, Indianapolis, Indiana.

Epidemic influenza types A and B viruses have been grown in the usual way in 11-day fertile eggs. The infected allantoic fluids are recovered following two days' incubation and then one day of chilling in the ice box. A wide variety of types of vaccine have been prepared from viruses grown in this way and having strong degrees of mouse virulence from 10^{-4} cc. to 10^{-6} ml. The most effective vaccine for Swiss mice is one preserved with "Merthiolate" (Sodium Ethyl Mercuri Thiosalicylate, Lilly) 1:20,000 and devitalized by incubating at 37°C. for seven days. In immunizing action this vaccine far exceeds that prepared with formalin and phenol.

Due to possibility of an impending epidemic of influenza and lack of time for preliminary trial of vaccine, we have prepared high-titered bivalent antiserum by intravenous immunization of rabbits with purified virus suspensions. This is prepared specifically for inhalation prophylaxis at a time when an epidemic is first appearing in the community. Methods of preparation of this antiserum were discussed and references made to very recent work of a similar nature conducted by American, British, and Russian workers.

WAR-TIME IMMUNIZATION. *W. A. Jamieson*, Lilly Research Laboratories, Indianapolis, Indiana.

The most recent methods of preparation of vaccines against cholera, plague, yellow fever and typhus fever were described. These vaccines are of particular interest for troops leaving the United States.

This laboratory has made its greatest contribution to the immunization program of the war effort in the preparation of typhus vaccine. Different forms of typhus vaccine preparation were presented, and the current fertile egg method dealt with in some detail. Methods of assay of typhus and other vaccines in the laboratory, also primary and re-immunizations as developed during the last year or two, were discussed.

RECENT DEVELOPMENTS CONCERNING THE ANAEROBIC BACTERIA AND THEIR ACTIVITIES, WITH PARTICULAR REFERENCE TO THE TETANUS AND GANGRENE ORGANISMS. *L. S. McClung*, Bacteriological Laboratories, Indiana University, Bloomington, Indiana.

The recent literature relating to the anaerobic bacteria was reviewed. Topics discussed included: Toxin production by the tetanus and gangrene organisms; use of toxoid as an immunizing agent; growth factors and the problem of synthetic media; chemoprophylaxis and chemotherapy; techniques for the isolation and identification of anaerobic spore-formers, etc.

FORTY-FIFTH MEETING

OF

THE SOCIETY OF AMERICAN BACTERIOLOGISTS

New York City, May 3rd, 4th, and 5th, 1944

(Wed., Thurs., Fri.)

The scientific program will include papers on the various phases of bacteriology and related immunological phenomena. In so far as restrictions will permit, topics relating to wartime problems in bacteriology will be emphasized.

Round table and panel discussions are being arranged and one or more general sessions of wide interest are anticipated. A copy of the preliminary program will be sent to all members of the Society and those who register at the meeting will receive a copy of the abstracts of all papers. These abstracts will be published in the May issue of the *Journal of Bacteriology*.

Abstracts of papers to be presented at the meeting must not exceed 250 words in length and must be received by the Chairman of the Program Committee on or before February 14th, 1944. Abstracts received after this date will not be accepted.

The various committees in charge of the meeting are as follows:

Program Committee

Chairman: L. S. McCLUNG, Bacteriological Laboratories, 302 Chemistry Building, Indiana University, Bloomington, Indiana

Vice-Chairman: COLIN M. MACLEOD

Publicity Committee

Chairman: COLIN M. MACLEOD, New York University, College of Medicine, New York, New York

Local Committee in charge of arrangements

Chairman: MARY B. HORTON, Sheffield Farms, 524 West 57th Street, New York, New York

Vice-Chairman: W. W. BROWNE, City College of New York, 139th Street and Convent Avenue, New York, New York

Division of Medical Bacteriology, Immunology and Comparative Problems

Chairman: L. A. JULIANELLE, Public Health Institute of The City of New York, Foot of East 15th Street, New York, New York

Vice-Chairman: JOHN G. KIDD, Rockefeller Institute, 66th Street and York Avenue, New York, New York

Division of Agricultural and Industrial Bacteriology

Chairman: ELIZABETH MCCOY, Department of Agricultural Bacteriology, University of Wisconsin, Madison 6, Wisconsin

Vice-Chairman: R. P. TITTSLER, U. S. Department of Agriculture, Bureau of Dairy Industry, Washington 25, D. C.

The meetings will be held at the *Hotel Pennsylvania* in New York City.

Reservations for rooms should be made with the hotel as soon as possible. Society members desiring low priced accommodations should return the official hotel reservation card at the earliest possible date. *Special rates* for graduate students may be had upon application to the hotel or to the Chairman of the Local Committee.

Exhibits will be under the general direction of C. Virginia Fisher, Warner Institute for Therapeutic Research, 113 West 18th Street, New York, New York, with whom all exhibitors should communicate before February 14th.

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